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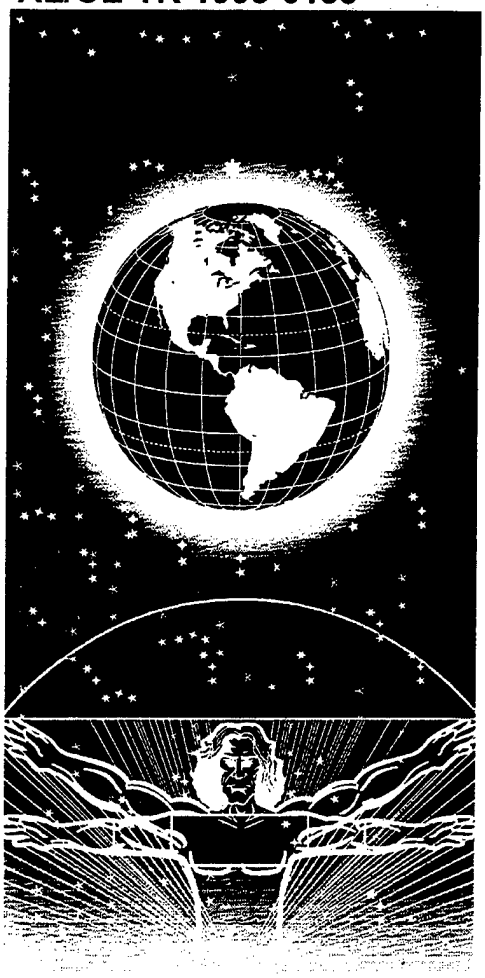

UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

1994 TOXIC HAZARDS RESEARCH UNIT (THRU) ANNUAL REPORT

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April 1995

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TECHNICAL REVIEW AND APPROVAL

AL/OE-TR-1995-0135

The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR

STEPHEN R. CHANNEL, Maj, USAF, BSC
Branch Chief, Operational Toxicology Branch
Air Force Armstrong Laboratory

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13. ABSTRACT (Maximum 200 words)

This reports presents a review of the activities of the Toxic Hazards Research Hunt (THRU) for the period 01 October 1993 through 30 September 1994. The THRU conducts descriptive, mechanistic, and predictive toxicology research and toxicological risk assessments to provide data to predict health hazards and to assess health risks associated with human exposure to chemicals and materials associated with military systems and operational environments. This report includes summaries of ongoing or completed research activities for major project efforts tasked by the Tri-Service Toxicology; highlights of the research support elements and conference activities of the THRU; and appendicies that describe the THRU organization and its publications and presentations. The majority of the report describes the progress attained in toxicological investigations on a wide variety of chemicals and materials to include trichloroethylene; Halon 1301 or 1211 replacement candidates iodotrifluoromethane, HFC-227ea, HFC-125, HCFC-123, and FC-218; and nitrate explosive and propellant candidates 1,3,5-trinitrobenzene, liquid propellant XM465, ammonium dinitramide, and 1,3,3-trinitoazetidine. The THRUalso summarized the research findings of select jet fuel studies conducted in the 1970s and 1980s, conducted research on lactational transfer modeling, and evaluated statistical methods for assessing military populations as a subgroup of the public at-large.

14. SUBJECT TERMS

Trichloroethylene	Trichloacetic acid	HCF-227ea	Halon 1301
dichloroacetic acid	Ammonium dinitrimide	HFC-125	Jet fuel JP-4
1,3,5-trinitrobenzene	Iodotrifluoromethane	HCFC-123	
Liquid propellant XM46	Ammonium dinitramide	FC-218	1,3,5-trinitoazetidine

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PREFACE

The 31st Annual Report of the Toxic Hazards Research Unit (THRU) presents research and research support efforts conducted by ManTech Environmental Technology, Inc. on behalf of the U.S. Air Force, the U.S. Army, and the U.S. Navy under Department of the Air Force Contract No. F33615-90-C-0532. This document represents the fourth annual report for the current THRU contract and describes accomplishments from 01 October 1993 through 30 September 1994.

Operation of the THRU under this contract was initiated on 16 January 1991 under Project No. 6302, "Occupational and Environmental Toxic Hazards in Air Force Operations," Work Unit No. 63020002, "Toxic Hazards Research." This research effort is cosponsored by the Army Medical Research Detachment, Walter Reed Army Institute of Research (WRAIR), Work Unit Nos. 611102.S15L and 612787.878L, and by the Naval Medical Research Institute Detachment/Toxicology (NMRI/TD), Work Unit No. M0096.004.0006, "Criteria for Exposure Limits in Navy Operational Environments."

The Toxicology Division, Occupational and Environmental Health Directorate, Armstrong Laboratory (AL/OET), Wright-Patterson Air Force Base, Ohio, provided the technical direction for this contract. Lt Col Terry A. Childress, Director of the Toxicology Division, served as the Contract Technical Monitor. That portion of the work effort sponsored by the Army was under the direction of LTC Roland E. Langford, Detachment Commander, and LTC Daniel Caldwell, Senior Scientist of the Medical Research Detachment. That portion of the work effort sponsored by the Navy was under the direction of the NMRI/TD Officer-in-Charge, CAPT David A. Macys, MSC, USN. Darol E. Dodd, Ph.D. served as the ManTech Environmental THRU Program Manager.

The contents and the preparation of this report represent the combined effort of the ManTech Environmental staff of the THRU and the staff of the ManTech Environmental Technical Publications and Graphics Department. Acknowledgment is made to Ms. Susie Godfrey, Betsy Huber, Suzanne Bornemann, Cindy Matthews, and James A. Miller for their assistance in the preparation of this report.

TABLE OF CONTENTS

SECTION 1

ABBREVIATIONS

μg	Microgram
μL	Microliter
μm	Micrometer
ACSL	Advanced Continuous Simulation Language
ADN	Ammonium dinitramide
AFSC	Air Force Development Test Center
AL/OET	Armstrong Laboratory, Toxicology Division
AL/OEVM	Armstrong Laboratory, Comparative Medicine Branch
ALD	Approximate lethal dose
ALKP	Alkaline phosphatase
ALT	Alanine aminotransaminase
ANOVA	Analysis of variance
AP	Ammonium perchlorate
AST	Aspartate aminotransaminase
AvGas	Aviation gasoline
BBDR	Biological based dose-response
BF	Body Fat
BHCM	Body Height in Centimeters
BUDS	Basic underwater demolition (team)
BW	Body weight
BWKG	Body Weight in Kilograms
C	Celsius
CF_3I	Trifluoroiodomethane
CFC	Chlorofluorocarbon
CH	Chloral hydrate
CHO	Chinese hamster ovary
cm	Centimeter
CNS	Central nervous system
CO_2	Carbon dioxide
CV	Coefficient of variation
DCA	Dichloroacetic acid
DFM	Diesel fuel marine
DMN	Dimethylnitrosamine

DMSO	Dimethylsulfoxide
DNB	1,3-Dinitrobenzene
DoD	Department of Defense
DOT	Department of Transportation
DPA	2,2,Dichloropropionic acid
EA	Exposure Assessment
ECD	Electron Capture Detection
ECG	Electrocardiogram
EEGL	Emergency exposure guidance level
EM	Electron microscopy
EMH	Extramedullary hematopoiesis
EMS	Ethyl methanesulfonate
EPA	U.S. Environmental Protection Agency
F	Farenheit
F-344	Fischer 344
FC-218	Octafluoropropane
FID	Flame ionization detector
ft	Foot
g	Gram
g/dl	Grams per deciliter
GC	Gas chromatograph(y)
GC/MS	Gas chromatography / mass spectrometry
GD	Gestation days
GGT	Gamma-Glutamyl transferase
GI	Gastrointestinal
GLP	Good Laboratory Practice
GSH	Glutathione
h	Hour
HAN	Hydroxylammonium nitrate
HCT	Hematocrit
HFC-125	Pentafluoroethane
HFC-227ea	1,1,1,2,3,3,3-Heptafluoropropane
HGB	Hemoglobin
HGPRT	Hypoxanthine-guanine phosphoribosyl-transferase
HMX	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
HPLC	High performance liquid chromatograph(y)

HSR	Health & Safety Representative
HTG	Thyroglobulin
Hz	Hertz
IMF	Induced mutant frequency
in.	Inch
ip	Intraperitoneal
IR	Infrared
IU/L	International units per liter
kg	Kilogram
L	Liter
LAN	Local area network
lb	Pound
LC	Lowest concentration
m	Meter
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MF	Mutant frequency
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mmHg	Millimeters of mercury
MN	Micronuclei
msec	Millisecond
N	Number
N_2	Nitrogen
NCE	Normochromatic Erythrocyte
NGM/ML	Nanogram per milliliter
NMRI/TD	Naval Medical Research Institute, Toxicology Detachment
NOAEL	No observable adverse effect level
NOEL	No observable effect level
NZW	New Zealand White
O.D.	Outer diameter
O_2	Oxygen
OHEA	Office of Health and Environmental Assessment

OSHA	Occupational Safety and Health Administration
p	Probability
PBPK	Physiologically based pharmacokinetic
PC	Personal computer
PCE	Polychromatophilic erythrocytes
PCE/NCE	Polychromatophilic erythrocyte / normochromatic erythrocyte
pg	Picogram
ppb	Parts per billion
ppm	Parts per million
psi	Pounds per square inch
QAA	Quality Assurance Associate
QAC	Quality Assurance Coordinator
QAU	Quality Assurance Unit
RBC	Red Blood Cells
RT_3	Reverse T_3
RTG	Relative total growth
SD	Standard deviation
SDFM	Shale-derived diesel fuel marine
SEALS	Sea, air, land (team)
sec	Second
SEM	Standard error of the mean
SF	Slope Factor
SFE	Solid Fuel Extinguishant
SIDS	Screening Information Data Set
TBARS	Thiobarbituric acid reactive substances
TBD	To be determined
TBG	Thyroxine-binding globulin
TCA	Trichloroacetic acid
TCE	Trichloroethylene
TCOG	Trichloroethanol glucuronide
TCOH	Trichloroethanol
TEM	Triethylenemelamine
TFA	Trifluoroacetic acid
TFT	Trifluorothymidine
TH	Total hydrocarbon
THRU	Toxic Hazards Research Unit

TK	Thymidine kinase
TMP	Thymidine monophosphate
TNAZ	1,3,3-Trinitroazetidine
TNB	1,3,5-Trinitrobenzene
TNT	2,4,6-Trinitrotoluene
TPH	Total petroleum hydrocarbon
TSCA	Toxic Substances Control Act
U/mL	Units per milliliter
v/v	Volume per volume
VC	Viable count
w/v	Weight per volume
WBC	White blood cell
WPAFB	Wright-Patterson Air Force Base
wt	Weight
XM46	Liquid Propellant formulation 1846

SECTION 2
INTRODUCTION

This report presents a review of the activities of the ManTech Environmental Technology, Inc., Toxic Hazards Research Unit (THRU), for the period 01 October 1993 through 30 September 1994. ManTech Environmental's THRU is an on-site, contractor-operated, United States Air Force, Army, and Navy multidisciplinary research program. The THRU conducts descriptive, mechanistic, and predictive toxicology studies and toxicological risk assessments to provide data to predict health hazards and to assess the health risks associated with human exposure to chemicals and chemical materials of interest to the military. The major goal of the THRU's research efforts is to contribute to safe military operations, including safe occupational and environmental conditions. An additional goal of the THRU is to advance the state-of-the-art in toxicology research and risk assessment techniques.

The THRU conducts research on a variety of materials that may range from pure chemicals to poorly defined mixtures. They include, but are not limited to fuels, lubricants, solvents, additives, components of explosives, propellants, paints, solvents, structural materials, training agents, and combustion products. Descriptive toxicology is used to identify toxic effects, target organs, and dose–response effects associated with different exposure routes, concentrations, and durations. Mechanistic toxicology is performed to determine toxicokinetics, mechanisms of action, and dynamics of expression of the toxic effects of the material of interest. Predictive toxicology involves the development, validation, and application of computer simulation models to describe quantitative dose–response relationships based on quantified input parameters such as exposure concentration, partition coefficients, respiratory rate, blood flows, rate of metabolite formation, rate of chemical excretion, and metabolic enzyme constants. These models are used to define target organ toxicity based on the tissue-specific dose and are used in intra- and interspecies extrapolation. Data generated via descriptive, mechanistic, and predictive toxicology studies are used together with interpreted literature data to produce human health hazard risk assessments.

In accordance with the THRU contract's Statement of Work and specific technical directives (study requests) provided by the Contract Technical Monitor, the THRU also coordinates toxicology conferences, expert workshops, and program reviews. Research support benefitting both THRU and government research efforts is provided in the areas of special test equipment design, fabrication, validation, modification, and maintenance; mathematics and biometry; computer systems management and programming; necropsy and histology techniques; toxicology information databases and library management; quality assurance; health and safety; and documentation and report preparation. The THRU's research support and administrative elements are integral to the quality, continuity, and productivity of its scientific research efforts.

The research and support efforts of the THRU represent a continuum of activities that may overlap two or more years depending upon the study scheduling and the extent of the research that is required. During this reporting period, studies performed in response to requirements of the Air Force included analyses of metabolites of trichloroethylene (TCE); development of alternatives for risk assessment of exposure to TCE; application of physiologically based pharmacokinetic models to cancer risk assessment for breast-fed infants; acute/subchronic toxicity testing, genotoxicity testing, cardiac sensitization screening, gas uptake kinetics, partition coefficient determinations, toxicokinetics and *in vivo/in vitro* metabolism studies of select Halon 1211 and Halon 1301 replacement candidates, such as HCFC-123, CF_3I, or HFC-227ea; and data analysis of jet fuel inhalation toxicity studies that were conducted in the 1970s and 1980s.

During this reporting period, the THRU received several study requests for toxicology research in support of the Army. The THRU conducted studies to evaluate the reproductive toxicity and/or genotoxicity potential of 1,3,5-trinitrobenzene (TNB), Liquid Propellant XM46, ammonium dinitramide and 1,3,3-trinitroazetidine. Additionally, mechanistic studies to determine the toxicity of nitrate-containing chemicals were initiated, and quality assurance support was provided for the teratologic evaluation of TNB on a study performed by Air Force personnel.

Toxic Hazards Research Unit technical directives that supported the Navy included continuation of a statistical methods study to define techniques for assessing the variability in sensitivity of the human population and subpopulations for developing a basis for altering safety factors used in risk assessments applicable to military populations. Additionally, a number of technical support efforts in animal necropsy, histology, and equipment design and fabrication were conducted.

During this reporting period, the THRU provided work effort in support of several toxicology conferences and workshops, including the series of annual toxicology conferences that have been coordinated by the THRU since 1965. The proceedings of the 1993 toxicology conference, "The Risk Assessment Paradigm After Ten Years: Science, Policy, and Practice Then, Now, and in the Future," were compiled by the THRU and distributed as a publication by the journal *Risk Analysis* (Vol. 14, No. 3, 1994). The 1994 toxicology conference, "Temporal Aspects in Risk Assessment for Noncancer Endpoints," was conducted in April at the Hope Hotel and Conference Center at Wright-Patterson Air Force Base (WPAFB). The proceedings of this conference have been compiled by the THRU for publication as a special issue of the journal *Inhalation Toxicology*. In August 1994, the THRU initiated its planning efforts for the 1995 toxicology conference, "Risk Assessment Issues for Sensitive Human Populations." The THRU provided support for the "1994 HazMat/Pollution Prevention Symposium," arranged for Aeronautical Systems Center/Environmental Management at WPAFB, and four additional workshops, including a TCE Science Workshop in Williamsburg, VA, and a Perchlorate Study Group Meeting at WPAFB.

The execution of the THRU contract Statement of Work involves the integrated effort of a multidisciplinary staff of scientists, research technicians, research support, and administrative personnel. Sections 3 through 8 of this report emphasize the technical activities of the THRU. Sections 9 and 10 present highlights of the conferences and research support activities, including research engineering, mathematics and statistics, pathology support, computer and electronic support, quality assurance, and health and safety. Section 11 of this report is a set of appendices that describe the THRU organization, its personnel, and its awards, publications and presentations.

Historically, the THRU has prepared annual reports on its research efforts since 1963. In general, these annual reports present summaries or highlights of the technical projects (study requests) that were directed by the Air Force, Army, and Navy. More descriptive reports on the THRU's research activities are prepared upon completion of study requests and are published as technical reports or peer-review publications (refer to "Products List for 1994" in Section 11). Technical reports also are prepared following the conferences and most workshops coordinated by the THRU. Copies of these technical reports are available from the National Technical Information Service or the Defense Technical Information Center.

3.1 ANALYSES OF DICHLOROACETIC ACID, TRICHLOROACETIC ACID, TRICHLOROETHANOL, AND TRICHLOROETHANOL GLUCURONIDE: METABOLITES OF TRICHLOROETHYLENE

W.T. Brashear, M.M. Ketcha[1], C.T. Bishop[2], and H.A. Barton

ABSTRACT

Trichloroethylene (TCE) has been identified as an environmental contaminant in groundwater. Trichloroacetic acid (TCA), dichloroacetic acid (DCA), trichloroethanol (TCOH), and trichloroethanol glucuronide (TCOG) have been identified as metabolites of TCE. Studies have shown that TCA and DCA can induce liver tumors in B6C3F1 mice. Methods for the analysis of these metabolites are important for conducting pharmacokinetic studies. TCA and DCA were derivatized to their methyl esters by dimethyl sulfate under acidic conditions and analyzed by gas chromatography with electron capture detection (GC/ECD). TCOG was hydrolyzed to free TCOH by β-glucuronidase or acid hydrolysis. TCOH was analyzed by GC/ECD after solvent extraction. Two important artifacts that can occur in analyzing the carboxylic acid metabolites of TCE are the conversion of TCA to DCA in fresh blood under acidic conditions and the loss of DCA from fresh liver homogenate. These artifacts can be prevented by freezing blood samples prior to analysis, and by deactivating liver enzymes with lead acetate.

INTRODUCTION

Trichloroethylene (TCE), a widely used degreasing and cleaning solvent, is an environmental contaminant commonly found in groundwater. Exposure to TCE is of concern because it has been found to be a rodent carcinogen (Bruckner et al., 1989). TCE is reported to be metabolized to chloral hydrate (CH), trichloroacetic acid (TCA), dichloroacetic acid (DCA), trichloroethanol (TCOH), and trichloroethanol glucuronide (TCOG) (Hathaway, 1980; Tanaka and Ikeda, 1968). DCA and TCA, like TCE, cause mouse liver tumors (Bull et al., 1990; DeAngelo et al., 1991).

Analytical methods have been developed to analyze biological samples for TCE and its metabolites (Breimer et al., 1974; Chen et al., 1993; Humbert and Fernandez, 1976; Ikeda et al., 1972,). However, new findings regarding the interconversion of DCA and TCA, and the *ex vivo* degradation of DCA are reported here. Precautions are needed to conduct proper quantitative analysis.

Previous methods have recognized the need to prevent the interconversion of metabolites. Breimer et al. (1974) used lead acetate to prevent the conversion of CH to TCOH in blood. They also reported that sulfuric acid will prevent the conversion of CH to TCOH by erythrocytes. Their

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recommendation is that blood samples be immediately mixed with lead acetate or sulfuric acid. Other methods have treated biological samples with sulfuric acid in order to extract DCA and TCA into an organic solvent (Goreki et al., 1990; Hathaway, 1980). It is known that the addition of acid will affect TCOH levels since acid has been shown to hydrolyze TCOG to free TCOH (Breimer et al., 1974; Garrett and Lambert, 1966). Therefore, acid-treated samples analyzed for TCOH will yield total TCOH since blood and urine have both free and conjugated TCOH (Goreki et al., 1990).

Our experiments show that TCA and DCA levels are also affected by the addition of sulfuric acid. This metabolite interconversion and evidence of *ex vivo* DCA metabolism are addressed in this paper. The method developed to analyze DCA, TCA, TCOH, and TCOG has been designed to minimize the interconversion and loss of analytes from biological samples.

MATERIALS AND METHODS

Laboratory Animals

Laboratory animals were obtained from Charles River Laboratories (Kingston, NY). Male B6C3F1 mice weighing 17–23g (6–7 weeks) were used for this study. Animals were exposed to 600 ppm TCE for 4 h, and placed into metabolism cages for 24-h urine collection. Unexposed mice were sacrificed by CO_2 asphyxiation for control tissue samples.

Chemicals & Equipment

TCOH Glucuronide. E. coli β-Glucuronidase (G-8271) and sodium acetate were obtained from Sigma Chemical Co. (St. Louis, MO). TCE (99+%, 25,642-0) and TCOH (99+%, T5,480-1) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Ethyl acetate was obtained from Mallinckrodt (Paris, KY) and acetic acid was obtained from J.T. Baker (Phillipsburg, NJ). Tissue samples were homogenized using a Tekmar Tissuemizer (Cincinnati, OH) and a Haake-Buchler vortex evaporator (Saddlebrook, NJ) was used to vortex and heat samples. Samples were analyzed on a Hewlett Packard 5890 gas chromatograph with a Hewlett Packard 7673A liquid autosampler (Hewlett Packard, Avondale, PA). The chromatograph was equipped with an electron capture detector and a 30 m × 0.53 mm Supelco Wax 10 column (Supelco, Belfonte, PA).

TCA, DCA, and Total TCOH. TCA (99+%, 25,139-9), DCA (99+%, D5,470-2), 2,2-dichloropropionic acid (94%, 29,115-3), and dimethyl sulfate (99+%, D18, 630-9) were obtained from Aldrich Chemical Company (Milwaukee, WI). Lead acetate was obtained from Mallinckrodt (Paris, KY). Sulfuric acid and hexane were obtained from Fisher Scientific (Fair Lawn, NJ). Samples were analyzed on a gas chromatograph with a liquid autosampler as previously described.

Assay Procedures

TCOH and TCOG. Urine was analyzed for free TCOH and TCOG. For free TCOH analysis, a 0.1 mL aliquot of urine was transferred to a 2 mL vial containing 0.2 mL of sodium acetate buffer (0.2 M sodium acetate buffer, adjusted to pH 5.7 via acetic acid). A second 0.1 mL aliquot of urine was incubated with 0.1 mL of β-glucuronidase (200 U/mL in sodium acetate buffer) for 4 h at 37 °C. This amount of β-glucuronidase can hydrolyze about 6 μg of TCOG to TCOH. After incubating with β-glucuronidase, 0.1 mL of 0.2 M sodium acetate buffer was added. A 1.0 mL aliquot of ethyl acetate was added to each sample. The samples were extracted on a shaking incubator for 30 min at 60 °C, and centrifuged at 2000 × g for 10 min. The ethyl acetate layer was removed and transferred to a 1.8 mL liquid autosampler vial and analyzed by GC. The chromatography conditions are shown in Table 3.1-1. Standards were prepared by extracting a solution containing 0.1 mL of control urine, 0.1 mL of 200 U/mL β-glucuronidase, and 0.1 mL of a TCOH standard.

TABLE 3.1-1. TCOH CHROMATOGRAPHY CONDITIONS

Injector Temperature	175 °C
Initial Temperature	70 °C
Initial Time	1 min
Rate	15 °C/min
Final Temperature	190 °C
Final Time	5 min
ECD Temperature	300 °C
Column	Supelco Wax 10, 25m × 0.53mm
Make Up Gas	5% Methane in Argon
Carrier Flow rate	6 mL/min

To establish conditions for enzymatic hydrolysis 24-h urine was collected from mice exposed to 600 ppm TCE for 4h. Urine was diluted 1:5, 1:10, and 1:50 with 0.2 M sodium acetate buffer (pH 5.7). A 0.1 mL aliquot of diluted urine was combined with 0.1 mL of 200 U/mL β-glucuronidase. Enzymatic incubations were quenched with 0.1 mL of 20% lead acetate at various time points and analyzed for TCOG.

DCA and TCA Analysis. Samples were analyzed for DCA and TCA by modification of a method by Maiorino et al. (1980). Tissue homogenates were prepared by placing 1g of tissue in 4 mL of cold saline and homogenizing the sample while on ice. Liver homogenate was inactivated by combining 0.1 mL of homogenate with 0.1 mL of 20% lead acetate. To prevent the conversion of

TCA to DCA, freshly collected blood samples were frozen at −20 °C for 24 h. A 0.1 mL sample of blood, urine, or tissue homogenate was placed in a 2 mL vial with 0.1 mL of water and 0.1 mL of 10 μg/mL 2,2-dichloropropionic acid (internal standard). The vials were placed on ice and allowed to cool for 30 min, then 0.5 mL of concentrated sulfuric acid and a 0.1 mL aliquot of dimethyl sulfate were added. The vials were capped and vortexed on a shaking incubator for 30 min at 60 °C. After vortexing the vials were allowed to cool and 1 mL of hexane was added. The samples were extracted on the incubator shaker for 60 min at 55 °C and centrifuged at 2000 × g for 10 min. The hexane layer was removed and analyzed by GC as described in Table 3.1-2. Standards were made from blood, urine, or tissue homogenates from control animals. A 0.1 mL aliquot was combined with 0.1 mL of 10 μg/mL 2,2-dichloropropionic acid (internal standard) and a 0.1 mL aliquot of an aqueous standard of DCA and TCA. The samples were derivatized and analyzed as previously described.

TABLE 3.1-2. DCA AND TCA CHROMATOGRAPHY CONDITIONS

Injector Temperature	175 °C
Initial Temperature	70 °C
Initial Time	15 min
Rate	15 °C/min
Final Temperature	190 °C
Final Time	5 min
ECD Temperature	300 °C
Column	Supelco Wax 10, 25m × 0.53mm
Make Up Gas	5% Methane in Argon
Carrier Flow Rate	6 mL/min

Preparation of Microsomes. Livers were perfused *in situ* with ice cold buffer containing 10 mM Tris, 154 mM KCl, pH 7.4, and quickly removed to cold buffer. Aliquots were homogenized in four volumes of the same buffer and centrifuged 20 min at 9000 × g to remove cellular debris, mitochondria, nuclei, and residual red blood cells. The resulting supernatant was centrifuged 1 h at 105,000 × g to separate the cytosolic fraction and microsomal fraction. Cytosolic supernatant was removed and the microsomal pellet was resuspended in buffer (above) to a concentration of approximately 15 mg protein/mL.

RESULTS

TCOH and TCOG

Control urine collected from B6C3F1 mice spiked with TCOH from 1 μg/mL to 25 μg/mL gave a linear standard curve for this range (r^2 = 0.99). TCOH had a retention time of 6.5 min, as shown in Figure 3.1-1. The extraction efficiency for TCOH was 95%, and was not affected by concentration. The limit of detection in urine was 0.1 μg/mL. The precision and accuracy of the method was evaluated from control mouse urine spiked at 5 μg/mL and 10 μg/mL TCOH. The precision was measured by analyzing spiked control samples which were made up fresh and run each day with a standard curve. The precision results are summarized in Table 3.1-3. Samples for accuracy determination were made from urine obtained from control mice. These were spiked with 8 μg/mL and 12 μg/mL TCOH and frozen at −20 °C for analysis at a later date. Individual samples were analyzed on different days using a freshly prepared standard curve. The accuracy results are summarized in Table 3.1-4.

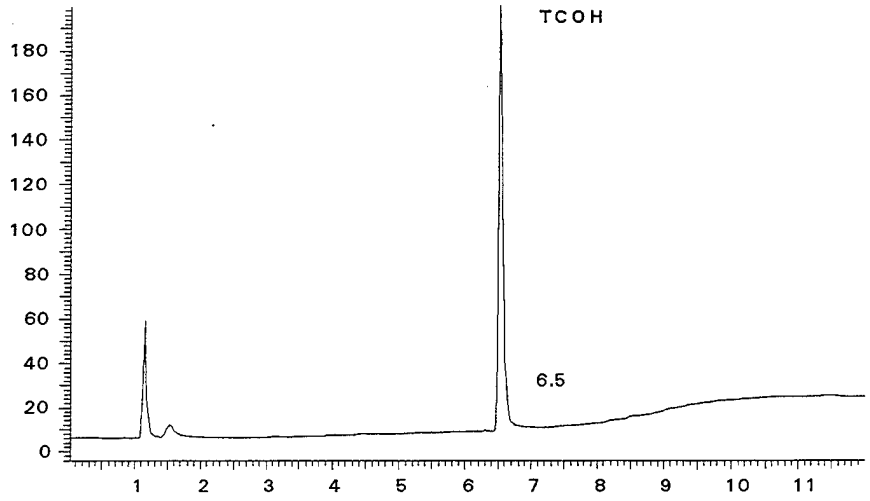

Figure 3.1-1. Chromatogram of a Urine Sample Containing 5 μg/mL TCOH. The retention time of TCOH was 6.5 min. Units of the y axis represent the detector response in millivolts.

TABLE 3.1-3. PRECISION OF TCOH ANALYSIS URINE SPIKED AT 5 μg/mL AND 10 μg/mL

	5 μg/mL	10 μg/mL
Mean	5.0 μg/mL	9.8 μg/mL
Std Dev	0.13 μg/mL	0.41 μg/mL
n	9	9
CV	2.5%	4.2%

TABLE 3.1-4. ACCURACY OF TCOH ANALYSIS URINE SPIKED AT 8 μg/mL AND 12 μg/mL

	8 μg/mL	12 μg/mL
Mean	7.9 μg/mL	12.4 μg/mL
Std Dev	0.63 μg/mL	1.4 μg/mL
n	7	7
CV	7.9%	11%

Urine was collected from mice exposed to 600 ppm TCE for 4 h. Urine diluted with sodium acetate buffer was analyzed for TCOG. The results, shown in Figure 3.1-2, indicate the enzymatic incubations are complete after 4 h with exposed urine diluted 1:50. This corresponds to a total TCOH level of 3000 μg/mL (±100 μg/mL, n = 4) in urine and 6 μg of total TCOH in the actual enzymatic incubation mixture. A sample of urine was analyzed without enzymatic hydrolysis for free TCOH. This was determined to be 30 μg/mL which indicates that 99% of the total TCOH in urine is TCOG.

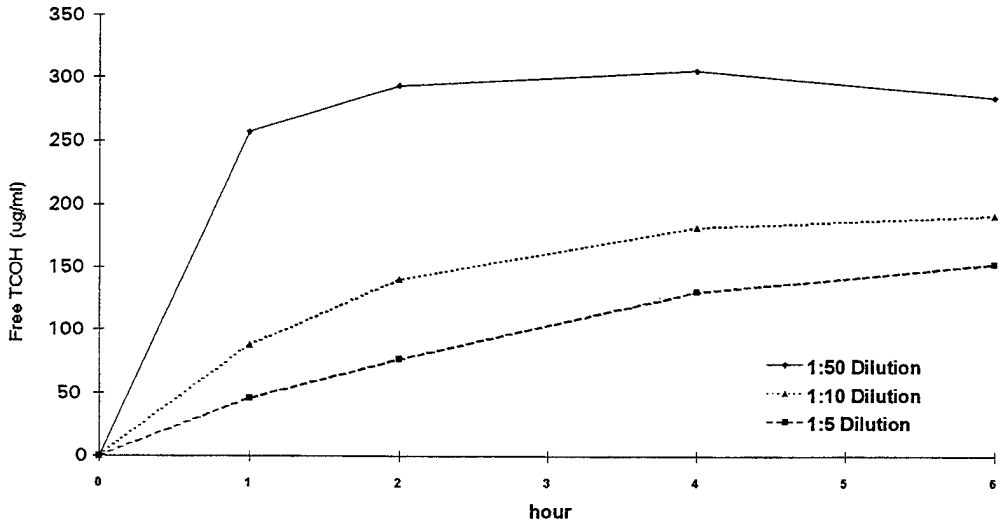

Figure 3.1-2. E. Coli β-Glucuronidase Incubations of 24-h Urine from Mice Exposed to 600 ppm TCE for 4 h.

To determine the effect of analyzing TCOH samples in the presence of strong acid, a sample of the same urine was incubated with sulfuric acid. A 100 µl aliquot of urine diluted 1:100 with 0.2 M sodium acetate buffer was combined with 0.5 mL of concentrated sulfuric acid, placed on an incubator shaker for 30 min at 60 °C, and extracted with ethyl acetate. This sample was determined to have 2800 µg/mL TCOH indicating that the TCOG glucuronide linkage is acid labile.

DCA and TCA Analysis

The assay developed for DCA and TCA had a limit of detection of 1.0 µg/mL for the analysis of 0.1 mL of blood or urine, and 1µg/g for a 0.1 mL sample of liver homogenate (1 g liver homogenized in 4 mL of saline). Standard curves were linear up to 25 µg/mL (r^2 = 0.99). As shown in Figure 3.1-3, the retention times of TCA, DCA, and DPA (internal standard) were 7.3, 8.3, and 4.6 min, respectively. From liver homogenate (1g of tissue in 4 mL of saline) and liver slice samples (6 mg/mL total protein), the reaction efficiency of the dimethyl sulfate derivatization was 95%, and the extraction efficiency was 50%.

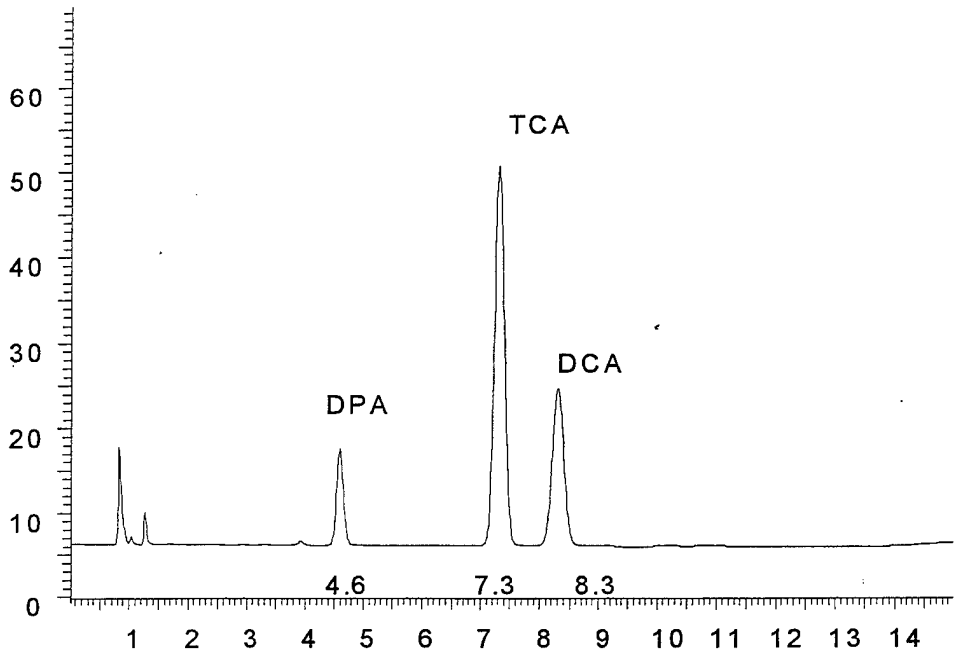

Figure 3.1-3. Chromatogram of 10 µg/mL TCA and DCA with Internal Standard DPA from Liver Homogenate. Retention times of DPA, TCA, and DCA were 4.6, 7.3, and 8.3 min, respectively. Units of the y axis is detector response in millivolts.

The precision of this method was also evaluated from microsomes (15 mg protein/mL) and liver homogenate (1g liver in 4 mL of saline). The precision was measured by analyzing spiked control samples which were made up fresh and run daily with a standard curve. The precision results are summarized in Table 3.1-5. Samples for accuracy determination were made from microsomes obtained from control mice. These were spiked with DCA and TCA and frozen at −20 °C for analysis at a later date. Individual samples were analyzed on different days using a freshly prepared standard curve over a period of 3 months. The accuracy results are summarized in Table 3.1-6.

TABLE 3.1-5. PRECISION OF DCA AND TCA ANALYSIS LIVER HOMOGENATE AND MICROSOMES SPIKED AT 10 μg/mL

	Liver Homogenate		Microsomes	
	DCA	TCA	DCA	TCA
Mean	9.8 μg/mL	10.7 μg/mL	9.8 μg/mL	10.2 μg/mL
Std Dev	0.40 μg/mL	0.75 μg/mL	0.69 μg/mL	0.87 μg/mL
n	14	14	34	34
CV	4%	7%	7%	9%

TABLE 3.1-6. ACCURACY OF DCA AND TCA ANALYSIS MICROSOME SAMPLES SPIKED AT 5 μg/mL

	DCA	TCA
Mean	5.0 μg/mL	5.1 μg/mL
Std Dev	0.65 μg/mL	0.57 μg/mL
n	16	16
CV	13%	11%

Samples of crude liver homogenate were spiked with DCA and TCA in order to develop standard curves. Liver homogenate from a control animal was spiked with DCA and TCA. From the analysis it was noted that DCA appeared to be lost from samples that were allowed to stand at room temperature for 30 min. To investigate this, a crude liver homogenate from control mice was spiked with different concentrations of DCA and allowed to stand at room temperature for 30 min. After standing, the samples were quenched with 0.5 mL sulfuric acid and derivatized with dimethyl sulfate. The results shown in Figure 3.1-4 indicate that DCA is metabolized by liver homogenate under these conditions.

Further testing indicated that the loss of DCA could be inhibited by pretreating the liver homogenate with 0.5 mL sulfuric acid or 0.5 mL of 20% lead acetate. Inactivated liver homogenate produced concentrations of DCA consistent with the expected values. A similar experiment conducted with TCA indicated that TCA is not metabolized by a crude liver homogenate under these conditions.

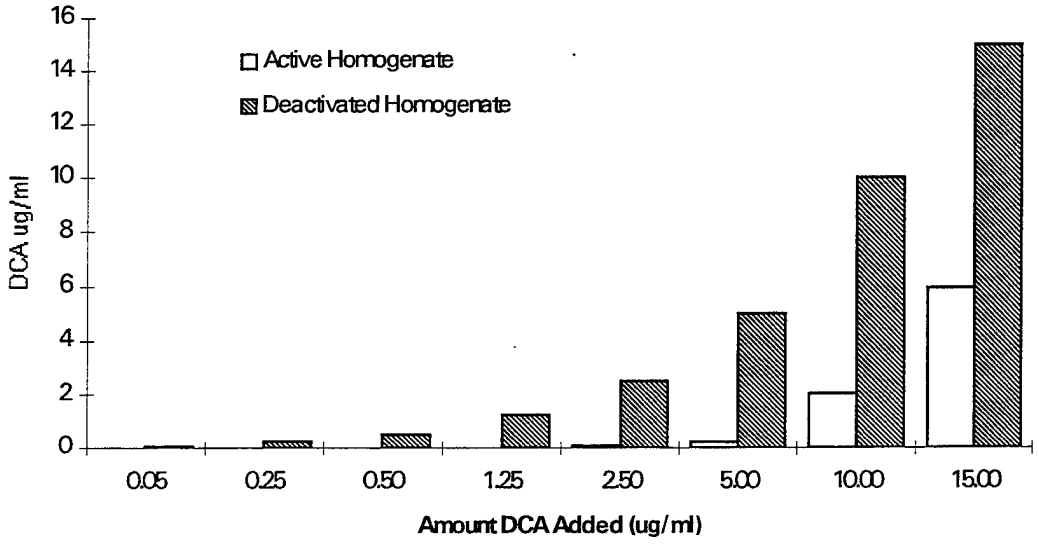

Figure 3.1-4. DCA Loss from Crude Liver Homogenate over 30 min at Room Temperature. Inactivated homogenate was inactivated with sulfuric acid.

Fresh blood samples spiked with TCA were found to convert TCA to DCA when treated with concentrated sulfuric acid. Fresh control blood was collected from mice and 0.1 mL aliquots were placed into tubes kept at room temperature which contained 0.1 mL of 50 μg/mL TCA. At time intervals of 0, 15, 30, and 60 min, 0.1 mL of concentrated sulfuric acid was added. Three hours after the blood was taken, the samples were derivatized with dimethyl sulfate and analyzed. The results, shown in Figure 5, suggest that TCA is converted to DCA by the action of concentrated sulfuric acid in freshly drawn blood. The conversion of TCA to DCA also occurred when 5%, 10%, 25%, and 50% sulfuric acid was added to fresh blood samples. The data in Figure 3.1-5 are a representative example of experiments in which TCA in fresh mouse blood converts to DCA under acidic conditions. The amount of conversion of TCA to DCA at the zero time point varied from zero to ninety percent.

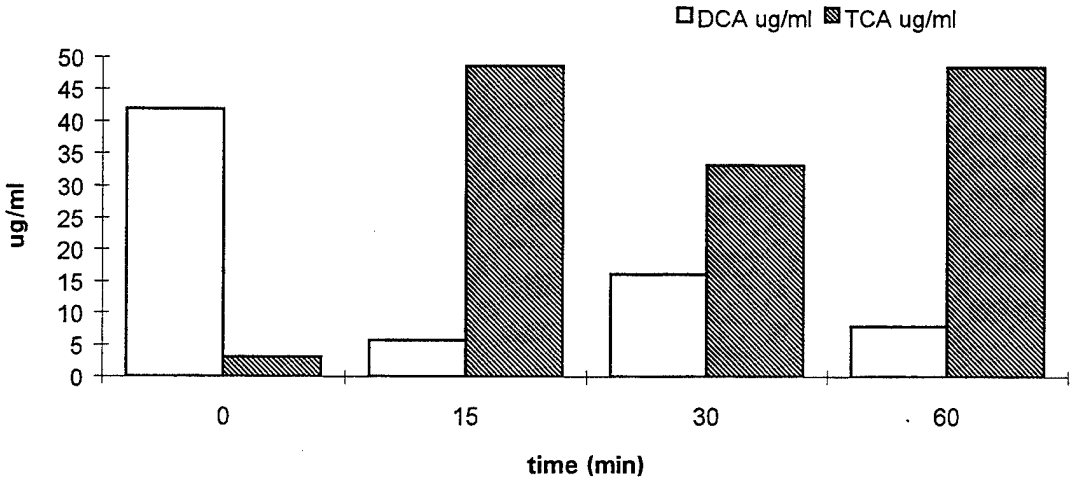

Figure 3.1-5. Conversion of TCA to DCA in Fresh Blood Samples from B6C3F1 Mice Spiked with 50 μg/mL TCA. Concentrated sulfuric acid was added at t = 0, 15, 30, and 60 min.

A 0.1 mL aliquot of fresh blood from a control animal was added to an ice cold vial which contained both TCA and 20% lead acetate. The presence of lead acetate did not prevent the conversion of TCA to DCA. However, if a TCA-spiked blood sample was allowed to be frozen for 24 h at −20 °C the conversion to DCA did not occur with the addition of concentrated sulfuric acid. After 24 h the observed TCA levels were consistent with the expected values. Sulfuric acid was added to fresh plasma from B6C3F1 mice which were spiked with TCA. This addition of 0.5 mL concentrated sulfuric acid did not convert any TCA to DCA.

To demonstrate the effect of treating blood samples from exposed mice with acid, mice were exposed to 600 ppm TCE for 7 h. Following exposure, mice were sacrificed and blood samples were obtained. Blood samples collected at various time points were immediately split into two portions. One portion was put into an equal volume of 10% sulfuric acid and kept on ice, the second was combined with an equal volume of distilled water and kept on ice. After collection, samples were stored at −20 °C for 24 h and analyzed for DCA and TCA. As shown in Figure 3.1-6, the samples immediately combined with acid contained DCA and TCA. Samples which were not combined with 10% sulfuric acid had higher concentrations of TCA, but no DCA.

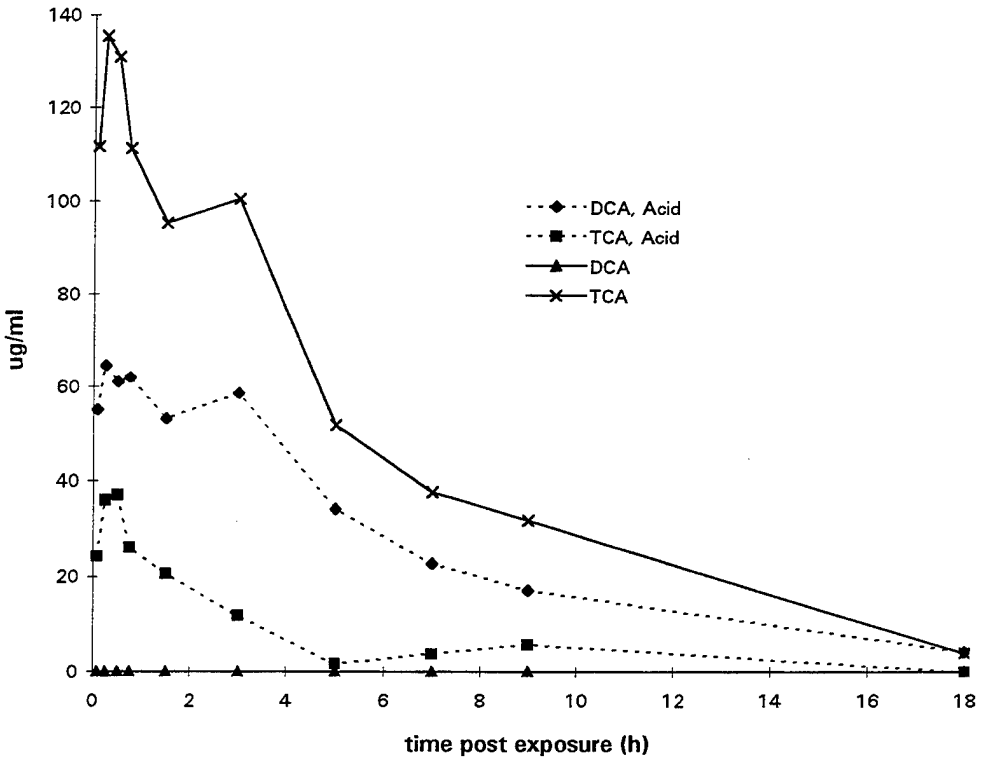

Figure 3.1-6. Conversion of TCA to DCA in Fresh Blood Samples from Mice Exposed to TCE. Samples at each time point were split into two portions. One portion was combined with 10% sulfuric acid (DCA, Acid and TCA, Acid), and the second with water (DCA, TCA).

DISCUSSION

These assays were developed for the determination of DCA, TCA, TCOH, and TCOG in biological samples. The analysis of TCOH and TCOG used an ethyl acetate extraction to measure free TCOH, and enzymatic hydrolysis followed by extraction to determine total TCOH. The difference represents the amount of TCOG. Treating urine from exposed mice with concentrated sulfuric acid has shown that the TCOG glucuronide linkage can also be hydrolyzed with acid. This is consistent with the reports of other investigators (Garrett and Lambert, 1966; Humbert and Fernandez, 1976) who have used acid hydrolysis for the analysis of total TCOH in urine. The glucuronide conjugate of TCOH has also been found in blood (Breimer et al., 1974; Gorecki et al., 1990). A previously published method (Breimer et al., 1974) for the analysis of total TCOH in blood used concentrated sulfuric acid to hydrolyze the glucuronide linkage and to inhibit the conversion of CH to TCOH. The experimental evidence of the interconversion of TCA to DCA in fresh blood indicates that this can affect the DCA and TCA levels.

The conversion of TCA to DCA in fresh blood is an important concern for the analysis of these metabolites of TCE. Published methods for the analysis of TCA (Breimer et al., 1974; Gorecki et al., 1990, Humbert and Fernandez, 1976) in biological fluids acidify samples with strong acid. This step is necessary in order to extract strong acids such as DCA (pKa 1.3) and TCA (pKa 0.7) into an organic solvent for methylation. Our work has shown that the acidification of fresh whole blood samples can lead to erroneous results. This has been demonstrated to occur *in vitro* with fresh blood spiked with TCA, as well as with fresh blood obtained from mice exposed to TCE. Conversion of TCA to DCA can be prevented by freezing the samples at −20 °C overnight before adding acid for derivatization. Plasma proteins do not appear to be responsible for the conversion of TCA to DCA since the reaction does not occur in fresh plasma from mice. The finding that pretreating blood with lead acetate does not prevent the TCA to DCA conversion also indicates that the conversion may not be due to enzymatic activity. The mechanism of the TCA to DCA interconversion is unknown. However, it is known that α-haloacetic acids can undergo electrocatalytic reductive dehalogenation (Rusling et al., 1990). This reaction is catalyzed by the reduced form of vitamin B_{12}, and does not require the electrochemical reduction of vitamin B_{12} α-haloacetic acid complex. It is possible that reduced metal porphyrins in fresh blood may be involved in reductive dehalogenation of TCA to DCA.

The loss of DCA from liver homogenate is another problem associated with the analysis of TCE metabolites. Homogenized liver samples need to be quenched with lead acetate as rapidly as possible prior to derivatization. The loss of DCA is due to *ex vivo* activity of liver enzymes (unpublished results). This can be inhibited by the addition of sulfuric acid or lead acetate. In Fischer-344 rats, DCA has been reported to be metabolized to glycolic acid, glyoxylic acid, oxalic acid, and carbon dioxide. The metabolism of DCA is saturable, and metabolites of DCA have a longer plasma half-life than DCA (Lin et al., 1993). Therefore, it is reasonable to expect the loss of DCA from biologically active samples.

SUMMARY

The results reported here show that it is critically important to evaluate the ability of metabolites to interconvert during analytical procedures, particularly when collecting data for pharmacokinetic analysis and assessing the metabolite(s) of TCE responsible for its carcinogenicity. Previous work has shown that erythrocytes can reduce CH to TCOH (Breimer et al., 1974). Our data has shown that the addition of acid to fresh blood samples can convert TCA to DCA. Blood samples need to be frozen overnight at −20 °C prior to the addition of acid for DCA/TCA analysis. The enzymatic activity in liver homogenate metabolizes DCA. Liver samples need to be deactivated with 20% lead acetate prior to DCA analysis.

REFERENCES

Breimer, D., H.C.J. Ketelaars, and J.M. Van Rossum. 1974. Gas chromatographic determination of chloral hydrate, trichloroethanol and trichloroacetic acid in blood and urine employing head-space analysis. *J. Chromatogr* 88:55–63.

Bruckner, J.V., B.D. Davis, and J.N. Blancato. 1989. Metabolism, toxicity, and carcinogenicity of trichloroethylene. *Crit. Rev. Toxicol.* 20(1):31–50.

Bull, R.J., I.M. Sanchez, M.A. Nelson, J.L. Larson, and A.J. Lansing. 1990. Liver tumor induction in B6C3F1 mice by dichloroacetate and trichloroacetate, in mouse liver. *Toxicology* 63:341–359.

Chen, X.M., C.E.Dallas, S. Muralidhara, V. Srivatsan, and J.V. Bruckner. 1993. Analysis of volatile C_2 haloethanes and haloethenes in tissues: sample preparation and extraction. *J. Chromatogr.* 612:199–208.

DeAngelo, A.B., F.B. Daniel, J.A. Stober, and G.R. Olson. 1991. The Carcinogenicity of dichloroacetic acid in male B6C3F_1 mouse. *Fundam. Appl. Toxicol.* 16:337–347.

Garrett, E.R. and H.J. Lambert. 1966. Gas chromatographic analysis of trichloroethanol, chloral hydrate, trichloroacetic acid, and trichloroethanol glucuronide. *J. Pharm. Sci.* 55(8):812–817.

Gorecki, D.K.J., K.W. Hindmarsh, C.A. Hall, and D.J. Mayers. 1990. Determination of chloral hydrate metabolism in adult and neonate biological fluids after single-dose. *J. Chromatogr.* 528:333–341.

Hathaway, D.E. 1980. Consideration of the evidence for mechanisms of 1,1,2-trichloroethylene metabolism, including new identification of its dichloroacetic acid and trichloroacetic acid metabolites in mice. *Cancer Letters* 8:263–269.

Humbert, B.E. and J.G. Fernandez. 1976. Simultaneous determination of trichloroacetic acid and trichloroethanol by gas chromatography. *Int. Arch. Occup. Environ. Health* 36:235–241.

Ikeda, M., H. Ohtsuji, T. Imamura, and Y. Komoike. 1972. Urinary excretion of total trichloro-compounds trichloroethanol, and trichloroacetic acid as a measure of exposure to trichloroethylene and tetrachloroethylene. *Br. J. Ind. Med.* 29:328–333.

Lin, E.L.C., J.K. Mattox, and F.B. Daniel. 1993. Tissue distribution, excretion, and urinary metabolites of dichloroacetic acid in the male fischer 344 rat. *J. Toxicol. Environ. Health* 38:19–32.

Maiorino, R.M., A.J. Gandolfi, and I.G. Sipes. 1980. Gas-Chromatographic method for the halothane metabolites, trifluoroacetic acid and bromide, in biological fluids. *J. Anal. Toxicol.* 4:250–254.

Rusling, J.F., C.L. Miaw, and E.C. Couture. 1990. Electrocatalytic dehalogenation of α-haloacetic acids by vitamin B_{12}. *Inorg. Chem.* 29:2025–2027.

Tanaka, S. and M. Ikeda. 1968. A method for determination of trichloroethanol and trichloroacetic acid in urine. *Br. J. Ind. Med.* 35:214–219.

3.2 DEVELOPING ALTERNATIVES FOR CANCER RISK ASSESSMENT: TRICHLOROETHYLENE AND ITS METABOLITES

H. A. Barton, D. J. Caldwell[1], S. R. Channel[2], and J. W. Fisher[2]

ABSTRACT

Chemical contamination of groundwater and soil is a national problem that consumes extensive technological and financial resources. Development of alternatives for cancer risk assessment can play an important role in making site remediation a health protective and cost effective activity. This project addresses trichloroethylene (TCE) as a case study for alternative risk assessment methods because it is one of the most widespread groundwater contaminants. TCE has been shown to be a carcinogen in rodents, but it appears to be a tumor promoter rather than a genotoxic initiator. Currently, dose–response values for cancer risk assessment are all derived using the linearized multistage model regardless of the likely mode of action of the chemical. This results in very low estimates of acceptable concentrations in groundwater, making remediation an expensive process. Alternative methods will be explored including development of physiologically based pharmacokinetic (PBPK) models for TCE and its metabolites and exploration of methods to address the pharmacodynamic processes of tumor promotion. This paper describes the overall plan and context for the project and reports the ongoing work.

INTRODUCTION

Chemical contamination of groundwater and soil is a national problem that consumes extensive technological and financial resources. Cleanup levels are determined on the basis of risk calculations, usually requiring extrapolation from laboratory animal studies. Determining acceptable levels for humans from animal studies is a conservative, policy-driven process that involves extrapolation and interpretation of scientific findings. Scientific uncertainties in this process are often compensated for by conservative assumptions that result in lower cleanup levels with inherent increased costs.

Current costs to clean up to the low ppb range in water and soil are in the hundreds of millions of dollars. Operating costs of a groundwater treatment system for a single plume at Wright-Patterson AFB, contaminated with high levels of trichloroethylene (TCE), are $1.3 million/year. TCE remediation at 31 Department of Defense (DoD) installations has cost $670 million to date, while work for all solvents has cost $2 billion. It has been estimated that for 125 Air Force sites with TCE contamination, raising the drinking water standard from 5 to 50 ppb would save $620 million.

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Trichloroethylene, tetrachloroethylene (PCE), and other volatile organics are priority groundwater contaminants for the DoD and the U.S. Environmental Protection Agency (EPA). These chemicals often greatly exceed current risk-based cleanup levels at Superfund landfill sites. This project is designed to further the development of newer risk assessment methodologies for developing reasonable health protective criteria for important groundwater contaminants for use in establishing cleanup requirements.

This project furthers the utilization of newer risk assessment methodologies for establishing cleanup criteria that incorporate more scientific information and minimize the use of conservative default assumptions. These methods would also be useful for other risk-based processes such as evaluating alternatives under consideration for pollution prevention purposes. TCE and its metabolites will be the chemicals used as case studies in this project due to the great importance of TCE as the most common groundwater contaminant in the country.

The four-step paradigm for risk assessment provides a framework for integration of scientific research and environmental policy analysis. Therefore, this project has been organized around the use of (1) hazard assessment, (2) exposure assessment (EA), (3) dose–response assessment (DR), and (4) risk characterization in the development of health based cleanup criteria.

Newer risk assessment methods include: physiologically based pharmacokinetic (PBPK) modeling to improve exposure and tissue dosimetry (EA & DR), biologically based dose–response (BBDR) modeling describing toxicity processes (DR), benchmark dose estimation using complete dose–response data (DR), and probability (Monte Carlo) analysis (EA & DR). These methods incorporate molecular toxicology into the risk assessment process. Alternative risk assessment approaches have been developed by researchers from the Toxicology Division, Occupational and Environmental Health Directorate, Armstrong Laboratory, (AL/OET), EPA, and academia.

The goals and objectives outlined below were developed by scientists from the participating agencies with modifications subsequent to the international TCE Science Workshop. Emphasis was given at that meeting to developing PBPK models for TCE and its metabolites (see Objective 2) and to evaluating the carcinogenicity of metabolites in relation to the parent chemical (Objective 3). Due to recent revisions to the EPA guidance for cancer risk assessment, a unique opportunity exists for developing risk assessment methods for TCE.

TCE Cancer Science Workshops

Revisions of EPA cancer risk assessment guidance have been drafted to incorporate modern knowledge about cancer mechanisms. Implementation of the proposed guidelines within the agency has been considered using a case study approach. This provided the framework for recent meetings considering the current science for TCE.

The Trichloroethylene Science Workshop held on 2–3 December 1993 was cosponsored by the Air Force, Trichloroethylene Issues Group, and EPA. It was preceded by a smaller research issues meeting held 31 November 1993 at WPAFB, OH. This first meeting included presentations and research discussions with the Triservices TCE research team. Presentations were made by Drs. R. Bull (Washington State), A. DeAngelo (EPA), T. Green (Zeneca, Ltd), J. Bruckner (U. Georgia), W. Dekant (Universität Wurzburg), and H. Barton (ManTech Environmental, WPAFB). Research plans of the Toxicology Division and other scientists were reviewed to develop useful collaborations and limit unnecessary duplication.

Nearly 40 scientists met in Williamsburg, VA, for the TCE Workshop, including leading researchers and policy analysts in the areas of epidemiology, metabolism, pharmacokinetics, tumor promotion, biological effects modeling, and risk assessment. Attendees included the toxicology researchers listed above and Drs. J. Popp (Sterling Winthrop) and J. Fisher (AL/OET). EPA attendees included the Office of Health and Environmental Assessment (OHEA) Deputy Director, Dr. J. Wiltse, Drs. J. Cotruvo (the Office of Pollution Prevention and Toxics), H. Gibb (OHEA), and J. Parker (OHEA). Developers of alternative risk assessment methods included H. Clewell (Crump Associates), and Drs. M. Andersen (EPA), and R. Connolly (Chemical Industry Institute of Toxicology). Industrial producers and users were also represented.

Discussion sections reviewed the wealth of data accumulated concerning TCE and its metabolites in the seven years since EPA prepared its last cancer risk assessment. The goal was to discuss issues and identify areas of agreement or disagreement to assist those with policy responsibilities in formulating their action plan.

Recent negative epidemiological studies strongly buttressed the assumption at the meeting that TCE has very weak, if any, activity in humans. One of the largest studies of workers at Hill AFB, UT, was presented by Dr. R. Spirtas (National Institutes of Health) (Spirtas et al., 1991). It was agreed that animal carcinogenicity for TCE and its two major metabolites to depend upon the species (rat, mouse). These findings, together with metabolism, clearly distinguish TCE from a classic genotoxic carcinogen such as vinyl chloride. The mode of action appears to be nongenotoxic tumor promotion, but activity of a minor glutathione (GSH) metabolite associated with male rat kidney tumors remains to be determined.

The meeting concluded with a review of EPA's draft guidance for cancer risk assessment and its application to TCE. This guidance uses a weight of evidence approach to incorporate the extensive database for TCE into the risk assessment process, rather than using a single default approach based

solely upon animal bioassays. It is highly likely that in the near future EPA will reconsider the risk for TCE using this guidance. Advice and assistance in carrying out this process will likely be provided by many of those who attended the workshop, including representatives of AL/OET. This would begin a process that, over the next few years, could affect site-specific risk assessments for TCE.

A TCE research exchange meeting was held on May 16, 1994, attended by researchers from the THRU, AL/OET, Drs. R. Bull and D. Stevens (Washington State), L. Lash (Wayne State), and J. Parker. Presentations and discussions were held concerning pharmacokinetics and pharmacodynamics in mouse liver and kidney of TCE and its metabolites, particularly DCA.

Ongoing and Proposed Research

The research effort described here is based upon the paradigm depicted below. This integrated risk assessment approach maximizes the utilization of scientific information facilitating environmental policy decision-making and implementation.

EXPOSURE → PHARMACOKINETICS → BIOLOGICAL EFFECTS → RISK ASSESSMENT

PROJECT GOAL

Develop innovative risk assessment methods that are applicable to common volatile organic water and air contaminants for use in development of scientifically defensible cleanup criteria.

SPECIFIC RESEARCH PLANS BY OBJECTIVES

OBJECTIVE 1: Evaluate new exposure assessment approaches to address the future use scenarios used to derive cleanup criteria.

1.1 Evaluate Utilization of Probability (Monte Carlo) Analysis for Future Use Exposure Scenarios for Volatile Organics in Water such as Drinking (Oral) or Showering (Inhalation)

Development of innovative dose–response assessments can alter exposure data needs. Traditional approaches are designed to use exposure concentrations averaged over a day or a lifetime, but biologically based dose–response modeling may address episodic and variable exposures.

Exposures to TCE are expected to occur predominantly through inhalation and oral pathways. Consumption of water containing TCE is the major expected oral route although, generally, people have been provided with other sources of potable water if TCE levels exceed the maximum contaminant level. Swimming in surface water containing TCE could also result in some oral intake. Inhalation of ambient air may result in exposure, but inhalation while showering may be more significant because TCE volatilizes from hot water. Extensive experimental and modeling efforts have already been directed towards the showering scenario for numerous chemicals (McKone, 1987).

Implementation of probability (Monte Carlo) analyses for describing exposure variations within a population will be investigated. Emphasis will be given to future use scenarios, since it is these, rather than the actual current use, that drive the development of cleanup criteria. At this time, actual current exposure appears minimal with drinking water supplies treated to meet the current maximum contaminant level of 5 ppb. Publication of the results in the peer-reviewed literature will provide supporting documentation for application of the findings to DoD site-specific risk assessments.

OBJECTIVE 2: Improve DOSE estimation across species (mouse, rat, and human) using PBPK modeling and supporting research.

2.1 Improve Quantitative Descriptions of the Metabolic Pathways and Uptake Mechanisms for TCE and its Metabolites in Mice, Rats, and Humans Using Appropriate *In Vivo* and *In Vitro* Laboratory Studies

2.1.A. Analytical methods capable of measuring all metabolites in the presence of other metabolites in biological samples including liver and blood.

Methods are available for measuring TCE, trichloroethanol (TCOH), TCOH-glucuronide (TCOG), and trichloroacetate (TCA) in blood and urine (Breimer et al., 1974). Currently analyses for TCE, TCOH, and TCA are routinely used in AL/OET laboratories. We are also implementing methods for TCOG, chloral hydrate (CH), and dichloroacetate (DCA).

Analytical difficulties are associated with CH and DCA in blood and liver. This is, in part, because these chemicals are rapidly metabolized in those tissues subsequent to removal from the animal. Our laboratory has shown that DCA also is metabolized in crude liver homogenate, and that the metabolism can be quenched by the addition of acid. Efforts to quench the *ex vivo* metabolism of CH and DCA have typically involved treatment with strong acid which may affect the concentration of other metabolites. This is a topic of ongoing research. The interconversion of metabolites prior to or during quenching could lead to erroneous data.

Dichloroacetate is of particular interest because it is a short-lived minor metabolite in liver, yet it is a potent inducer of mouse liver tumors. Chloral hydrate is the product of TCE oxidation, however, it is so rapidly metabolized that it is present at very low levels in liver and blood. It is an important metabolite to model quantitatively, because it is feasible to expose humans to CH for purposes of obtaining pharmacokinetic data (see 2.1.c). This presents an invaluable opportunity to collect data that can be used to validate models in humans and rats for the products of CH metabolism (see 2.1.c).

Extensive analytical work at the Toxicology Division has resulted in a modified method for measurement of the two strong acid metabolites, TCA and DCA (W. Brashear and M. Ketcha, personal communication). This method uses derivitization to form a methyl ester followed by gas chromatography with electron capture detection (GC/ECD). The limits of detection for this method are 0.5 mg/ml. It would be ideal to have a much lower detection limit, but the current method seems to be a reasonable compromise given the difficulty of working with this strong acid (pKa = 1.3).

2.1.B. Metabolic pathways in mice.

Extensive research has identified metabolites of TCE (Bruckner et al, 1989). Chloral hydrate is the initial product of TCE oxidation by cytochrome P450, but it is highly unstable. Major metabolites include TCOH, TCOG and TCA. A minor product is DCA. Several other minor metabolites have been identified arising from conjugation of glutathione to TCE and additional metabolism of all the metabolites. Of these chemicals, TCE, CH, TCA, and DCA have all been shown to cause liver tumors in mice. Some of the GSH derivatives have been shown to be mutagenic raising concerns about their potential for toxicity. Studies for this task are designed to fill gaps in the existing literature and ongoing research in other laboratories.

Ongoing research has been focussing on the metabolism of TCE measured with subcellular fractions to obtain estimates of enzymatic rates. This work will be extended to measure rates of TCE disappearance in liver slices, a more complete *in vitro* system. The data from these studies will be critical for implementing PBPK modeling using both *in vivo* and *in vitro* studies for comparisons across species (see section 2.1.c).

The metabolism in liver and kidney of other metabolites is under investigation. The disposition in several *in vitro* systems will be evaluated. These systems include liver slices, isolated hepatocytes, and subcellular fractions (microsomes and cytosol). Isolated hepatocytes of rat and mouse are routinely

produced in AL/OET laboratories and liver slice methodology has been implemented. With this method, liver architecture, the proportion of cell types, cell-to-cell communication, and cell membrane transport processes are maintained, and normal cellular metabolism remains largely intact. Subcellular fractionation is a widely used technique in several of the participating laboratories.

The metabolism of DCA by subcellular fractions from livers of mice, rats, and humans is being determined. This work is intended to (1) help determine if the lack of DCA formation from TCE and CH *in vitro* is due to rapid degradation and (2) specify the appropriate structure for PBPK modeling. It has been speculated in the literature that the metabolism of DCA required cytochromes P450 and involved reductive dehalogenation. Using supernatant from a 9000 × g spin (S9), it was found that P450 inhibitors did not decrease DCA degradation (W. Brashear and M. Ketcha, personal communication). Further studies using subcellular fractions showed that DCA was degraded by cytosol with little or no activity in microsomes or mitochondria (J. Lipscomb and D. Mahle, personal communication). DCA degradation occurred in cytosols from several tissues; activity from highest to lowest was found in liver > lung > intestine >> kidney = muscle.

As required for the development of PBPK modeling, additional metabolism work is expected to analyze CH, TCOH, TCOG, and GSH conjugates. The GSH conjugates are formed in the liver and other tissues but undergo unique metabolic pathways in the kidneys. The studies described are critical for providing information that will be used subsequently for linking the PBPK models for each metabolite.

2.1.C. Species comparisons.

Quantitative risk assessment for TCE may be done using epidemiological studies or positive findings from the laboratory rodent bioassays. In the latter case, extrapolation across species is required. It will be addressed in several parts because TCE-induced tumors are dependent upon exposure route and exposed species. Research efforts will focus on appropriate species, tissues, and metabolic steps. For instance, liver tumors in mice are clearly associated with CH, TCA, and DCA; no liver tumors were seen in rats. On the other hand, kidney tumors in rats may be associated with the GSH pathway, but no kidney tumors were seen in mice.

These differences in tumor response may be due to species-specific metabolism, other pharmacokinetic factors, or the subsequent toxicity process (pharmacodynamics). Comparative metabolism studies will begin to address this issue and help determine how to appropriately develop a biologically based dose–response model.

Each of the steps discussed above for metabolism in mouse will then be compared with metabolism in rats and humans. Ongoing studies with liver slices from human tissues are determining the kinetics of DCA metabolism. Subsequent studies will be required for metabolism of the parent TCE and each metabolite. These studies are critical to providing estimates for PBPK modeling of humans and for improving methodologies for cross species extrapolation. Results will be published in the peer–reviewed

literature to provide appropriate documentation for utilization in risk assessments by EPA and state agencies.

2.2 Expand PBPK Modeling Beyond its Current Strength with Volatile Organics, to Better Address Water Soluble Compounds, Such as Metabolites of TCE, that are Toxicologically Relevant

2.2.A. Develop model for trichloroethanol and chloral hydrate.

Chloral hydrate (also known as trichloroacetaldehyde) and TCOH represent chemicals with properties intermediate between the volatile hydrophobic compounds (e.g., TCE) and water soluble nonvolatiles (e.g., TCA and DCA). Both are fairly water soluble, but also volatile. Chloral hydrate is used medicinally and is the subject of research and modeling in humans (Strategic Environmental Research and Development Program — EPA). Limited pharmacokinetic studies with both chemicals in mice will need to be supplemented. More extensive studies are available in the literature using other species. Critical issues for modeling will include partitioning into tissue, metabolism (see 2.1), and enterohepatic recirculation. Protocols have been approved for carrying out studies including partition coefficient determination and i.v. dosing pharmacokinetics. Determination of CH and TCOH partition coefficients using an *in vitro* method for nonvolatile compounds has recently been completed (J. Creech, B. Garrity, and C. Seckel, personal communication).

2.2.B. Develop models for chloroacetic acids.

Two chloroacetic acids, DCA and TCA, are metabolites of TCE. Both are fully charged at physiological pH and, therefore, are water soluble nonvolatiles. Each chemical induces tumors in the livers of mice. Both chemicals are the subject of extensive biological research funded by EPA and the American Water Works Research Foundation. Therefore, these chemicals are excellent choices for extending PBPK modeling beyond its common use for volatile organic. Pharmacokinetic studies in mice and *in vitro* experiments to estimate model parameters will support model development. Critical issues will be cellular uptake, metabolism (see 2.1), binding to serum proteins, and excretion. Protocols have been approved for carrying out studies including partition coefficient determination and i.v. dosing pharmacokinetics. Initial studies of the distribution of DCA and TCA between red blood cells and serum, and binding to serum proteins have been carried out and additional work will continue as required (S. Neurath and H. Barton, personal communication).

2.3 Evaluate the Pharmacokinetic Competency of Carcinogenic Metabolites of TCE to Account for the Cancer Induced by the Parent Compound

2.3 A. Link the models for TCE and its metabolites.

TCE toxicity, particularly its carcinogenicity, arises from its metabolites. Thus, linking the PBPK models for the individual metabolites to each other and parent TCE is a critical step. In at least one case metabolism gives rise to two compounds, CH goes to TCOH and TCA. Correctly modeling this split will be dependent upon *in vitro* data (see 2.1), particularly for developing human models. Detailed experimental approaches will be designed once modeling of the metabolites has significantly advanced.

An extensive initial draft of a model linking PBPK models for TCE and its metabolites with pharmcodynamic and statistical models has been prepared (J. Byczkowski, personal communication). This model has been fit to selected data sets for TCE, CH, TCA, and DCA. The model helps to demonstrate the complexity of this task, the strengths and weaknesses of the available data for estimating paramater values, and the need for additional modeling of individual metabolites and pharmacodynamic processes.

2.3.B. Evaluate existing TCA and DCA cancer studies.

Once models for the metabolites and TCE in mice have been linked, it will be possible to address the critical question of accounting for the carcinogencity of TCE based upon one or both of these two metabolites. This information will be obtained by modeling existing chronic bioassays in which mice were exposed to TCA or DCA in drinking water.

OBJECTIVE 3: Improve RESPONSE comparisons across species using alternative modeling approaches and supporting research.

3.1 Evaluate the Correlation of Biomarkers of the Cancer Process with Production of Radicals During TCE Metabolism

Few studies have addressed the mechanisms by which TCE produces tumors in the livers of B6C3F1 mice, although it has been suggested that TCE acts as a tumor promoter. Neither TCE nor its metabolites, TCA and DCA, are mutagenic in standard assays (Greim et al., 1975; Waskell, 1978). Neither direct TCE-DNA adduction nor indirect effects, such as strand breaks, have been consistently observed in mice (Bergman, 1983; Nelson and Bull, 1988). Indirect effects clearly do not occur in rats (Chang et al., 1992).

The literature consensus suggests that DNA's effect, if a necessary step in the tumor-forming process, may be a consequence of lipid peroxidation by TCA and/or DCA (Bull et al., 1990; Larson and Bull, 1992a; Larson and Bull, 1992b). Generation of chemical free radical species, particularly carbon centered free radicals, may give rise to the histopathology observed with TCA or DCA exposure and is

consistent with other reports linking lipid peroxidation and tumorigenicity. Preliminary work at AL/OET has demonstrated that TCE exposure to B6C3F1 mouse liver explants does produce radical species.

Recent studies of the mechanism of carcinogenesis have focussed on the central role of cell proliferation in chemically induced tumors. Selected cell populations must increase in number beyond normal growth constraints to produce tumors. Moolgavkar (1990) and others have proposed a two-stage model incorporating cell proliferation rates and programmed cell death (apoptosis) for predicting risk of cancer. TCE has been shown to increase cell proliferation and/or DNA synthesis (Elcombe et al., 1985). DCA has been shown to produce a rapid increase in DNA synthesis which shows a dose–response relationship (Sanchez and Bull, 1990). Other researchers have reported cellular proliferation activity to be confined to putative preneoplastic lesions (DeAngelo et al., 1993). DCA produces marked cytomegaly and subsequent focal necrosis. In contrast, TCA treatment produces a uniform distribution of labeled cells, which was identical to control animals. Even at the high dose, necrotic areas were of such low frequency (2/20 sections) that is was impossible to determine if they were treatment-related. Although cytomegaly was observed, it was mild compared to that of DCA.

The effects of both compounds at Day 5 and TCA at Day 14 did not correlate with replicative DNA synthesis. Increased repair synthesis is one possible explanation of this phenomena; i.e., cells were not dividing, rather, they were repairing DNA. This would be consistent with results from this laboratory showing a prolonged G2M cell cycle phase, the period for DNA repair activity, in a rat hepatocyte cell line treated with TCA (Channel and Hancock, 1992).

Work by Phelps and Pereira (1993) suggests that precancerous (initiated) cells may remain sensitive to TCA or DCA for a longer period than uninitiated cells, which could result in their clonal expansion. This is a sort of "promotion" effect which has been demonstrated for TCA, although it is much weaker than that of a classical promoter such as phenobarbital (Parnell et al., 1986). This effect may be due to the production of radical species, which have been linked to tumor promotion (Kensler and Trush, 1984).

TCA and DCA act to produce tumors through processes that appear to be compound specific. Although cell proliferation must be a common event for both, the magnitude and time course differences cited previously suggest that other factors, such as their generation and subsequent metabolism (see 2.1), must be accounted for in order to produce a complete mechanistic explanation of the tumorigenicity of the parent compound.

Several studies are proposed for completion of this task. These will determine if radicals produced during TCE metabolism correlate with DNA damage, lipid peroxidation, or cell proliferation. Such effects may be biomarkers of the tumor development process that could then be incorporated in biologically based dose-response modeling. Studies of biomarkers will also provide support for the involvement of TCA, DCA, or both, in the induction of tumors by TCE.

3.1.A. Correlation study: *in vivo*, 60-day TCE exposure(s) in B6C3F1 male mice.

This project will determine correlations among radical formation, DNA oxidant damage, lipid peroxidation, and cell proliferation in liver. Because the definitive tumor chronic bioassay in the B6C3F1 mouse was performed using TCE in corn oil gavage, that exposure route will be employed here, initially. The exposure period of 60 days was selected as the optimum for observing both lipid peroxidation and cell proliferation changes.

DNA oxidant damage, as measured by 8-hydroxy-deoxyguanosine, will be quantitated in isolated nuclei to minimize interference from non-nuclear DNA. Lipid peroxidation will be measured by analysis of thiobarbituric acid reactive substance assay. Tissue sections will be assessed for cell proliferation by immunohistochemistry and/or *in situ* hybridization staining. Each endpoint will be evaluated for time course and dose–response. Correlations between each endpoint will be evaluated to establish the linkage among these mechanistic endpoints. Comparisons of this data with published accounts of TCA or DCA treatment effects may establish one of these metabolites as a predominant influence in TCE toxicity.

The gavage dosing for the 60-day study was completed. Male B6C3F1 mice were exposed to 400, 800, or 1200 mg/kg/day TCE in corn oil, corn oil alone (vehicle control), or no exposure (controls). Animals were euthanized at multiple time points ranging from 0 to 56 days. Shortly before sacrifice animals were injected i.p. with spin trap to capture radicals present. Livers were removed from animals for use in analyzing several different endpoints described above. These analyses are currently ongoing (S. Channel, K. Geiss, J. Kidney, J. Latendresse, and G. Randall, personal communication).

Chemical absorption, distribution, and elimination can vary depending on the route by which it enters the body. Thus, the route of exposure may play a significant role in both the character and quantity(s) of metabolites produced from a given dose of TCE (see 2.3). Consequently, this study may be repeated using other exposure routes such as inhalation, drinking water suspension, or non-corn oil vehicle (e.g., Emulphor) gavage. These studies would determine to what extent the linked mechanistic scheme is influenced by route of entry (metabolism) considerations.

3.2 Develop Conceptual Frameworks for BBDR Modeling to Integrate Pharmacokinetics (Exposure, Dose, Tissue-Dose) with Markers of Early and Late Biological Responses

3.2.A. Evaluate use of the benchmark dose approach for BBDR modeling of tumor promoters.

Current EPA risk assessments for the cancer endpoint have assumed the chemical could be genotoxic (i.e., DNA damaging) and a nonthreshold low dose extrapolation was appropriate. Current scientific literature clearly demonstrates that many chemicals act as tumor promoters rather than genotoxic initiators. Several possible approaches for risk assessment of tumor promotors are currently under consideration by EPA and others. These include clonal growth models (e.g., Moolgavkar-Venzon-

Knudsen model) and *de novo* biological effects models, such as that proposed for dioxin. Another approach would be to utilize the benchmark dose method.

The applicability of the benchmark dose method for tumor promotion will be evaluated. Issues of the appropriateness of this method for this endpoint, implications of background exposures to other genotoxic and nongenotoxic chemicals, and applicability to different classes of promoters will be considered. After consideration of the general applicability of the method, TCE will be used as a case study to evaluate the strength of the evidence that it acts as a promoter and application of the benchmark dose method. Comparisons to other methods will also be made, particularly the default linearized multistage model.

Initial work has focused upon the application of the benchmark dose to noncancer effects because this is the area to which it is most frequently applied (S. Das and H. Barton, personal communication). A review of the literature on noncarcinogenic effects of TCE identified liver alterations as the most widely reported and best documented toxic endpoint at lower concentrations. TCE is, by and large not toxic to male or female reproductive systems or to developing fetuses. However, limited recent data suggest it may increase the incidence of cardiac malformations. Comparison of the results of application of the benchmark dose method and the traditional no-observed-adverse-effect-level method is underway.

3.2.B. Evaluate the use of biomarkers in modeling correlations of TCE metabolism and biological effects.

Another aspect of the proposed modeling will be to explicitly extend the PBPK models into pharmacodynamic modeling of lipid peroxidation, a potential causitive factor in cell damage and tumor promotion by TCE. B6C3F1 mice will be exposed to various concentrations of TCE by inhalation in a controlled atmosphere chamber. Ethane is produced in the mouse as a consequence of lipid peroxidation, and thus may serve as a non-invasive indicator for the formation of radicals during metabolism. Animals will be removed at time points, and tissues (blood, liver) will be analyzed for each metabolite, CH, TCA, DCA, and TCOH, as well as residual TCE. In addition, thiobarbituric acid reactive substances (TBARS, see 3.1.a) will be measured. Metabolism rate estimates will then be correlated to the ethane and TBARS data to refine the model. The mathematical description of the linked metabolism and lipid peroxidation will be validated by reconciling model predictions with actual laboratory sample data.

OBJECTIVE 4: Propose new risk characterization strategies for utilization in development of cleanup criteria.

4.1 Evaluate the Implications of Alternate Dose–Response Modeling Methods for Acceptable Risk Levels

4.1.A. Evaluate the implications of species- and tissue-specific tumors for risk assessment.

TCE, like many other chemicals that are not genotoxic initiators, causes tumors in different tissues in each species. The finding of tumors in multiple tissues and species is a criteria that EPA uses for indicating that the chemical is more likely to cause cancer in humans. This interpretation was based upon experience with genotoxic initiators, which frequently induced DNA damage in many species and often in more than one tissue. However, in the absence of data supporting genotoxicity, the finding of tissue and species specificity of tumors should be evaluated differently.

This task will involve reviewing the literature and defining general guidelines for evaluating tissue and species specificity of tumors. TCE will be used as a key case study, but other volatile organics may provide additional case studies. Publication in the peer-reviewed literature will raise this issue in the risk assessment community and provide supporting documentation for EPA to reevaluate this issue under its new cancer risk assessment guidelines.

4.1.B. Evaluate the implications of alternate dose–response models for acceptable risk levels.

Acceptable exposure levels are determined by a range of scientific and policy factors, including legal language. In particular, they are dependent upon the methods used for exposure and dose–response assessment. As newer risk assessment approaches are implemented it is necessary to revisit the question of what the acceptable risk level should be.

The traditional methods for developing criteria for chemicals assume that there is a threshold or level below which no significant effects are expected. This was formalized as the Hazard Index or Margin of Safety approachs. These define methods for developing and applying chemical specific criteria. The assumption of a threshold was not considered valid for genotoxic carcinogens by analogy with the effects of radiation. This led to the common application of the linearized multistage model for estimating cancer risks for individuals and populations. In this context, the concept of *de minimus* risk levels developed at which there is some risk, albeit acceptably small.

Determining the level of acceptable risk has come to be called "risk management" and involves many factors, such as economics, technical feasibility, and site-specific characteristics, that are outside the scope of this project. This task will prepare a draft DoD position paper describing the implications of current scientific knowledge and risk assessment methods in the setting of acceptable risk levels. This document is anticipated to be applicable to a range of chemicals, in addition to TCE.

4.2 Recommend Provisional Remediation Goals for TCE to DoD and Draft Suggestions to EPA for TCE Risk Assesment under New EPA Guidelines

Development of cleanup criteria uses standard risk assessment procedures. To estimate risk, the chemical concentration must be estimated, and then used with the products of the exposure and dose-response assessments as shown here.

Fate and Transport		Exposure Assessment		Dose–Response Assessment		Risk Characterization
Chemical Concentration in Media	×	Exposure Pathway	×	Toxicity Value	=	Risk

To estimate cleanup criteria the equation is reversed:

$$CC = R / (E \times T)$$

This requires defining an acceptable risk level (see 4.1), defining potential future site use and exposure scenarios (see 1.1), and deriving dose–response criteria (see 2 & 3). All of these combine science and policy, though the first two are particularly dependent upon policy decisions. Cleanup criteria or provisional remediation guidance will be proposed for TCE in water and air, based upon selected exposure scenarios (see 1.1) and dose–response criteria (see 2 & 3). In addition, comments will be provided to the EPA concerning risk assessment for TCE under their new cancer risk assessment guidance.

REFERENCES

Bergman, K. 1983. Interactions of trichloroethylene with DNA *in vitro* and with RNA and DNA of various mouse tissues *in vivo*. *Arch. Toxicol.* 54:181–193.

Bruckner, J.V., B.D. Davis, and J.N. Blancato. 1989. Metabolism, toxicity, and carcinogenicity of trichloroethylene. *Crit. Rev. Toxicol.* 20:31–50.

Breimer, D.D., C.J. Ketelaars, and J.M. van Rossum. 1974. Gas chromatographic determination of chloral hydrate, trichloroethanol, and trichloroacetic acid in blood and in urine employing head-space analysis. *J Chromatog* 88:55–63.

Bull, R.J., I.M. Sanchez, M.A. Nelson, J.L. Larson, and A.J. Lansing. 1990. Liver tumor induction in B6C3F1 mice by dichloroacetate and trichloroacetate. *Toxicology* 63:341–359.

Chang, L.W., F.B. Daniel, and A.B. DeAngelo. 1992. Analysis of DNA strand breaks induced in rodent liver *in vivo*, hepatocytes in primary culture, and a human cell line by chlorinated acetic acids and chlorinated acetaldehydes. *Environ. Mol. Mutagen.* 20:277–288.

Channel, S.R. and B.L. Hancock. 1992. Cell cycle and growth effects of trichloroacetic acid in WB344 cells. *In Vitro Toxicol.* 5(4):241–250.

DeAngelo, A.B., F.B. Daniel, J.A. Stober, and G.R. Olson. 1991. The carcinogenicity of dichloroacetic acid in the male B6C3F1 mouse. *Fundam. Appl. Toxicol.* 16:337–347.

Elcombe, C.R., M.S. Rose, and I.S. Pratt. 1985. Biochemical, histological and ultrastructural changes in rat and mouse liver following the administration of trichloroethylene: Possible relevance to species differences in hepatocarcinogenicity. *Toxicol. Appl. Pharmacol.* 79:365–376.

Greim, H., G. Bonse, Z. Radwas, D. Reichert, and D. Henschler. 1975. Mutagenicity *in vitro* and potential carcinogenicity of chlorinated ethylenes as a function of metabolic oxirane formation. *Biochem. Pharmacol.* 24:2013–2017.

Kensler, T.W. and M.A. Trush. 1984. Role of oxygen radicals in tumor promotion. *Environ. Mutagen.* 6:593–616.

Larson, J.L. and R.J. Bull. 1992a. Metabolism and lipoperoxidative activity of trichloroacetate and dichloroacetate in rats and mice. *Toxicol. Appl. Pharmacol.* 115:268–277.

Larson, J.L. and R.J. Bull. 1992b. Species differences in the metabolism of trichloroethylene to the carcinogenic metabolites trichloroacetate and dichloroacetate. *Toxicol. Appl. Pharmacol.* 115:278–285.

McKone, T.E. 1987. Human exposure to volatile organic compounds in household tap water: The indoor inhalation pathway. *Environ. Sci. Techol.* 21:1194–1201.

Moolgavkar, S.H., ed. 1990. Two mutation models for carcinogenesis: Relative roles of sometic mutations and cell proliferation in determining risk. *Scientific Issues in Quantitative Cancer Risk Assessment* (Birkhauser, Boston, pp.136–152).

Nelson, M.A. and R.J. Bull. 1988. Induction of strand breaks in DNA by trichloroethylene and metabolites in rat and mouse liver *in vivo. Toxicol. Appl. Pharmacol.* 94:45–54.

Parnell, M.J., L.D. Koller, J.H. Exon, and J.M. Arnzen. 1986. Trichloroacetic acid effects on rat liver peroxisomes and enzyme-altered foci. *Environ. Health Perspect.* 69:73–79.

Phelps, B. and M.A. Pereira. 1993. Effect of dichloroacetic acid and trichloroacetic acid on cell proliferation in liver and precancerous lesions of B6C3F1 mice. Poster, Annual Toxicology Conference, Wright-Patterson AFB, OH.

Sanchez, I.M. and R.J. Bull. 1990. Early induction of reparative hyperplasia in the liver of B6C3F1 mice treated with dichloroacetate and trichloroacetate. *Toxicology* 64:33–46.

Spirtas, R., P.A. Stewart, J.S. Lee, D.E. Marano, C.D. Forbes, D.J. Grauman, H.M. Pettigrew, A. Blair, R.H. Hoover, and J.L. Cohen. 1991. Retrospective cohort mortality study of workers at an aircraft maintenance facility. I. Epidemiological results. *Brit. J. Ind. Med.* 48:515–530.

Waskell, L. 1978. A study of the mutagenicity of anaesthetics and their metabolites. *Mutat. Res.* 57:141–153.

3.3 APPLICATION OF PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL TO CANCER RISK ASSESSMENT FOR BREAST-FED INFANTS

J.Z. Byczkowski and J.W. Fisher[1]

ABSTRACT

The risk assessment process predicts the chances of adverse health effects that the toxicant possibly can do to the target organism under expected conditions of exposure. Regulators chose among several mathematical approaches to estimate the risk, but in each case it was necessary to link the dosemetrics of the toxicant with its predicted health effect. In this paper, a computer program is described that allows us to link a physiologically based pharmacokinetic (PBPK) model for tetrachloroethylene (PCE) in the lactating mother with the estimate of extra cancer risk for breast-fed infants, according to the U.S. Environmental Protection Agency (EPA) methodology. When inhaled by a lactating woman, PCE may partition into breast milk and may be transferred to the breast-fed infant. We have developed and experimentally validated a PBPK model for lactational transfer of PCE in rats, including a quantitative description of a milk compartment and the nursing pup. Subsequently, the model has been scaled to describe human physiology, and was validated with literature data for human cases of PCE exposure. Finally, we linked the dosage predictions of the PBPK model with equations used by EPA to estimate the cancer risk from PCE. The model predictions are in good agreement with both the measured values and those reported in the literature for exposure to PCE. This comparison confirms the usefulness of PBPK modeling in risk assessments.

INTRODUCTION

Toxicological research has been carried out traditionally through animal experiments and experimental data interpretation. Results of laboratory experimentation are used to quantify the toxicity by relating effects to the dose level. Toxicokinetic studies are used to determine the behavior of toxicant in organisms, and thus, what dose level of a toxicant may be expected in the organism and how it is distributed among the tissue compartments. The risk assessment process is used to predict the adverse health effects that the toxicant possibly can bring about to the human organism, usually at dose levels below the experimentally observable range. To perform risk assessment, it is necessary to determine the exposure of a particular human population to the toxicant. This determination may be especially difficult in the case of risk assessment of infants. Regulators have chosen several mathematical approaches to estimate risk, but in each case it is necessary to link the dosemetrics of the toxicant with its predicted health effect. Physiologically based pharmacokinetic (PBPK) models have proven particularly useful,

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through computer simulations, in defining the dosemetrics and in mathematical description of toxicokinetics.

In this paper, a computer program is described that allowed us to link a PBPK model for tetrachloroethylene (PCE) in the lactating mother with a theoretical estimate of extra cancer risk for breast-fed infants, according to the U.S. Environmental Protection Agency (EPA, 1989) methodology.

The chlorinated hydrocarbon, PCE, is a volatile organic solvent extensively used for dry-cleaning and degreasing. The agent has exhibited carcinogenic effects in experimental animals (ACGIH, 1993) and some U.S. agencies are considering its possible carcinogenic potential for humans (CDHS, 1991).

MATERIALS AND METHODS

Hardware and Software Specifications

A PBPK model was written in Advanced Continuous Simulation Language (ACSL), a FORTRAN-based language (Mitchell and Gauthier Assoc., 1987), and simulations were performed using SIMUSOLV software with optimization capabilities (DOW Chemical Co., Midland, MI; Steiner et al., 1990) on a VAX/VMS mainframe computer (VAX8530, Digital Equipment Corp., Maynard, MA).

Computational Methods and Theory

Toxicokinetics is the branch of toxicology that deals with movements and changes of toxicant in the organism, and thus can be used to describe the dose level of a toxicant expected in the organism and how it is distributed among the tissue compartments. There are significant differences between the classical pharmacokinetic approach of linear compartmental models and computer-assisted PBPK modeling.

In classical toxicokinetics, the concentration of a toxicant in a one-compartment system is calculated in the following way (Figure 3.3-1): the change in the amount of toxicant (dA) over time interval (dt) is proportional to the amount present in the compartment (A). Therefore, if the volume of distribution (V) stays constant, the concentration (C) is described by a similar relationship as the amount (Figure 3.3-1). Integrating this simple proportionality equation, one may calculate the concentration of toxicant at a given time. The constant of proportionality (k), usually referred to as the elimination rate constant, is inversely related to the half-life ($t_{1/2}$; Figure 3.3-1).

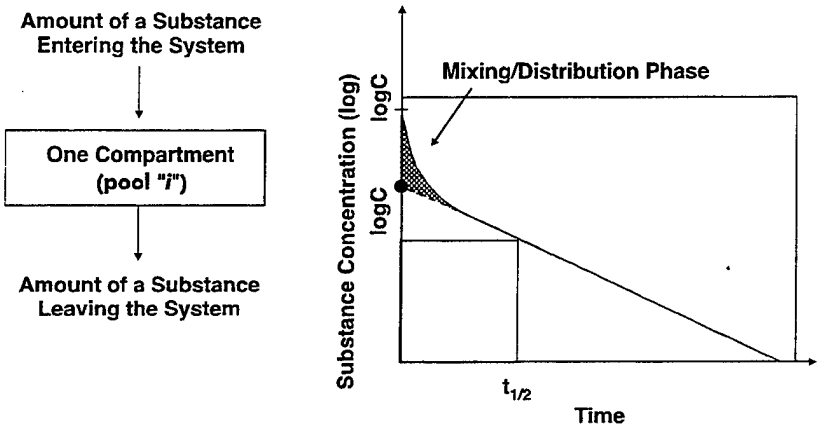

Figure 3.3-1. Basic Concepts of Classical Pharmacokinetics for One-Compartment Linear System.

$-dC/dt = k \times C$

$C(t) = C_0 \times \exp(-k \times t)$

$\log C(t) = \log C_0 - k - t/2.303$

$t_{½} = 0.693/k$

Where: × - multiplication.

C(t) - concentration of a substance (mg/L) in the compartment at time t.

C_0 - theoretical concentration (mg/L) at t = 0, assuming that a substance mixes into its total volume of distribution instantaneously.

k - the rate constant of a substance elimination (1/h), $t_{1/2}$ = half-life (h).

This basic model becomes more complicated when additional compartments are considered simultaneously. For the multicompartment linear system, the concentration in the "i-th" compartment (C_i) will be a sum of exponential terms of the form:

$$C_i = A_i e^{-at} + B_i e^{-bt} + \ldots + N_i e^{-Gt}$$

where each term represents a partial contribution to the total concentration in the "i-th" compartment.

One disadvantage of the classical approach is that experimental resolution of these biexponential, triexponential, etc., equations becomes progressively difficult. Another disadvantage is that it requires interpretation of several abstract parameters that are usually not directly available for experimental physical measurements and are often counter-intuitive (e.g., intercompartmental transport rate constants, compartmental volumes, volume of distribution, clearance, half-life, etc.).

These interpretations are not required for PBPK modeling since all compartments of the system are physiologically defined (Figure 3.3-2). A basic assumption in PBPK is that either blood flow to the tissue or diffusion are limiting the substance delivery. Because the substance is retained by the tissue according to its tissue/blood partition coefficient (PT), which may be measured *in vitro*, the concentration of the substance in venous blood, leaving the tissue (CVT), during the equilibration phase is lower than the concentration in arterial blood (CA). Therefore, the rate of change of substance in tissue (RAT), is given by a simple difference between concentration in blood entering and exiting the tissue (CA-CVT) multiplied by the blood flow (QTC) (Figure 3.3-2).

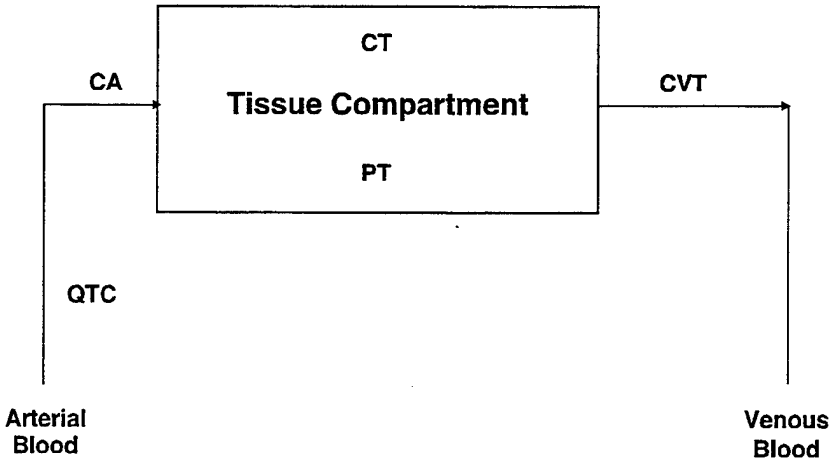

Figure 3.3-2. Basic Concepts of Physiologically Based Pharmacokinetic (PBPK) Modeling.

$RAT = QTC \times (CA - CVT)$

$AT = \int_0^t RAT\ dt$

$CVT = AT/(VTC \times PT)$

$CT = AT/VTC$

Where: × - multiplication.
RAT - rate of change of amount in tissue (mg/h).
QTC - fractional blood flow to tissue (L/h).
CA - concentration in arterial blood (mg/L).
CVT - concentration in blood leaving tissue (mg/L).
AT - amount in tissue (mg).
VTC - volume of tissue (L).
PT - partition coefficient tissue/blood (ratio).
CT - concentration in tissue (mg/L).

By integrating this equation over a given time, one can calculate the amount of substance present in tissue (AT) and, therefore, if the actual volume of tissue (VTC) is known, one can calculate the concentration of substance in the tissue (CT) at any time (Figure 3.3-2).

Multicompartmental PBPK models are built by interlinking several tissue compartments with arterial blood flow as an input and venous blood as an output (Ramsey and Andersen, 1984). The difference between classical and PBPK modeling approaches becomes apparent in the multicompartmental systems. PBPK models use experimentally verifiable physiological values for ventilation rate and blood flows to the organs and the physicochemical affinity (solubility) of the toxicant to particular bodily fluid or tissue. The solubility of a chemical in a tissue may be experimentally measured at steady state by its distribution (partitioning), for instance, between blood and air, liver and blood, milk and blood, and so on, and is called a partition coefficient. Metabolism of the substance can be measured and expressed in a way analogous to the Michaelis-Menten description of enzymatic activity. In addition, PBPK models employ

physically real volumes of organs, expressed as fractions of the body weight, that may be allometrically scaled up or scaled down, according to the size of the individual.

Thus, PBPK models require experimental determination of basic physiological parameters: blood flows, ventilation rates, organ volumes; physicochemical parameters: blood/air, and tissue/blood partition coefficients (Gargas et al., 1989); and biochemical parameters: apparent Michaelis-Menten constant of substance metabolism, pseudo-maximal velocity or first-order metabolism rate for its biotransformation (Gargas et al., 1986).

PBPK models are useful for mathematical description of pharmacokinetics and determination of biologically active doses in tissues which may be used for risk assessment. It is also possible, using PBPK models, to extrapolate between high and low concentrations, high and low body weight, and between different species.

Figure 3.3-3 depicts the concept of the relationship between cancer risk and a dose of toxicant (Figure 3.3-3a) along with the problems in extrapolation from high to low dose (Figure 3.3-3b). Usually, experiments on animals provide some data for carcinogenic effects of high, and occasionally, of moderate doses of toxicants. Unfortunately, there is usually no experimental confirmation of the dose–effect relationship for low doses of toxicant relevant to human exposure (Figure 3.3-3a). Simply, it is impossible to measure the effect in animals exposed to very low doses. What usually can be done in this situation is to extrapolate cancer risk from high to low exposure doses (Figure 3.3-3b). However, there is no universal consensus as to how to extrapolate the extra cancer risk from high to low exposure doses and different regulatory agencies use different approaches and recommend different algorithms. None of the methods used to date has been demonstrated to be scientifically and mechanistically accurate.

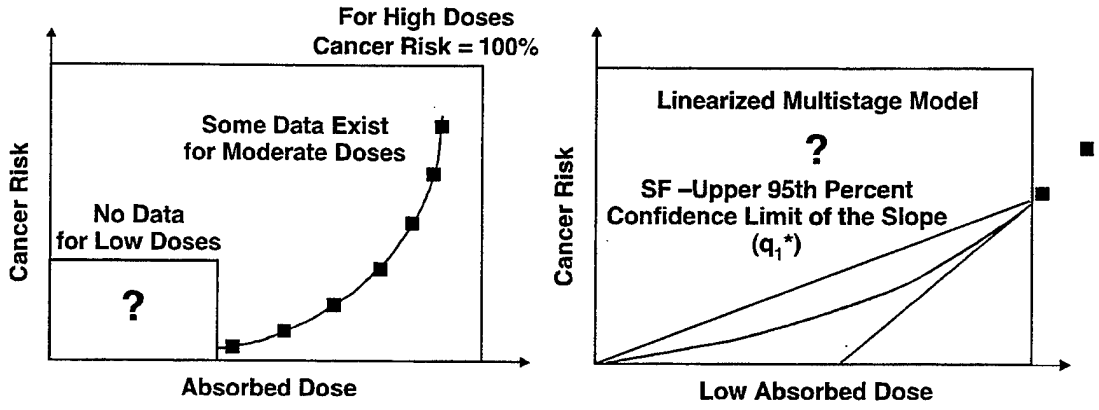

Figure 3.3-3. (a) Relationship between Cancer Risk and Absorbed Dose of Toxicant. (b) Problems with Cancer Risk Extrapolation from High to Low Exposure Doses.

In this program we followed the EPA methodology linking the low dose exposure to PCE with extra cancer risk by a slope factor (SF), calculated as an upper 95th percent confidence limit according to the linearized multistage model (Figure 3.3-b). The equation linking extra cancer risk (ECRI or TCRI) with a daily absorbed dose of PCE (IDM or TOTINF) is calculated simply by the product of slope factor (SF calculated for oral exposure to PCE (CDHS, 1991), dose and years of exposure, divided by expected life span of the infant (assumed to be 70 years):

$$ECRI = SF \times IDM \times Y/70 \text{ and } TCRI = SF \times TOTINF \times Y/70$$

where: $\times$ is multiplication, SF is the upper 95th percent confidence limit of the slope of the dose–response curve, expressed as $(mg/kg/day)^{-1}$, and Y represents years of exposure (EPA, 1989).

RESULTS

Program Description

Figure 3.3-4 shows a block scheme of the compartments of the PBPK model developed for lactational transfer of PCE. Initially, the model was built and then experimentally validated in nursing female rats and rat pups exposed to PCE via mother's milk. The physiological parameters were either measured experimentally in rats in our laboratory, or were taken from the literature (Byczkowski et al., 1994a,b,c). Once the model was satisfactorily calibrated in rats, we scaled it up to reflect human physiology (Byczkowski and Fisher, 1994).

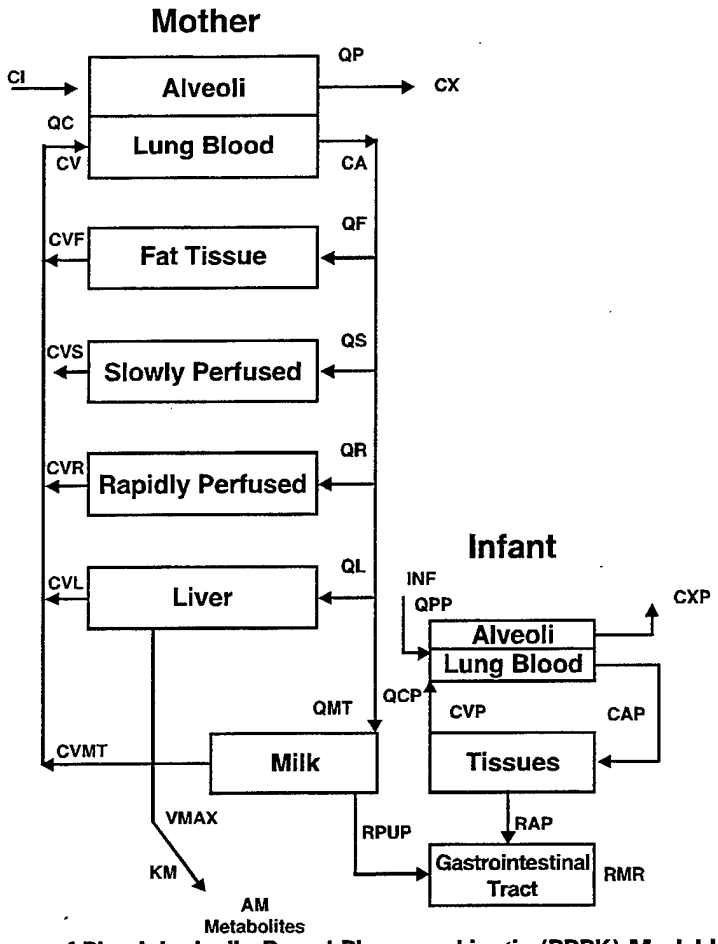

Figure 3.3-4. Scheme of Physiologically Based Pharmacokinetic (PBPK) Model Used to Simulate Lactational Transfer of PCE in Nursing Rats and Humans. (Byczkowski et al, 1994: Byczkowski and Fisher, 1994, 1994a, 1993).

Where: CI - concentration in inhaled air (mg/L).
QP - alveolar ventilation rate adjusted for body weight (L/h).
CX - concentration in exhaled air (mg/L).
QC - cardiac output adjusted for body weight (L/h).
CV - concentration in mixed venous blood (mg/L).
CA - concentration in arterial blood (mg/L).
CVF - venous concentration leaving the fat tissue (mg/L).
QF - blood flow to fat (L/h).
CVS - venous concentration leaving the slowly perfused tissues (mg/L).
QS - blood flow to slowly perfused tissues (L/h).
CVR - venous concentration leaving the rapidly perfused tissues (mg/L).
QR - blood flow to rapidly perfused tissues (L/h).
CVL - venous concentration leaving the liver tissue (mg/L).
QL - blood flow to liver (L/h).
QPP - alveolar ventilation rate in infant adjusted for body weight (L/h).
INF - concentration in air inhaled by infant (mg/L).
CXP - concentration in air exhaled by infant (mg/L).
QMT - blood flow to the mammary tissue (L/h).

(caption continued on next page)

QCP - cardiac output in infant adjusted for body weight (L/h).
CVP - concentration venous blood in infant (mg/L).
CAP - concentration in arterial blood in infant (mg/L).
CVMT - venous concentration leaving the mammary tissue (mg/L).
V_{MAX} - pseudo-maximal velocity of PCE metabolism (mg/h).
RPUP - elimination rate for PCE from milk to infant (mg/h).
RAP - the rate of gastrointestinal absorption of PCE in infant (mg/h).
KM - apparent Michaelis-Menten constant for PCE metabolism (mg/L).
RMR - the rate of gastrointestinal tract loading with PCE in infant (mg/h).
AM - the amount of PCE metabolized (mg).

For well-stirred tissue compartments, with one mass input (arterial blood) and one output (venous blood), such as fat, slowly perfused and rapidly perfused tissues, as well as infant solid tissues, the toxicant mass transfer is given by the differential equation:

$$dA_i/dt = Q_i(CA - CV_i)$$

$$CV_i = C_i/P_i$$

$$C_i = A_i/V_i$$

where: subscript $_i$ indicates the "i-th" compartment, A_i is the amount in "i-th" compartment, Q_i is the blood flow through the "i-th" compartment, CA is the arterial concentration, CV_i is the venous concentration leaving the "i-th" compartment, C_i is a concentration in the tissue in the "i-th" compartment, V_i is the volume of the "i-th" compartment, P_i is the tissue/blood partition coefficient for the "i-th" compartment.

For lung compartments with two mass inputs (mixed venous blood and inhaled air) and two outputs (arterial blood and exhaled air), at steady state the amount in alveolar air is in equilibrium with the amount in lung blood:

$$QP(CI - CX) = QC(CA - CV)$$

$$CX = CA/PB$$

where: QP is the air flow through the lungs (alveolar ventilation rate), CI is the concentration in inhaled air, CX is the concentration in alveolar air, CA is the arterial concentration (leaving the lungs), PB is blood/air partition coefficient, QC is the blood flow through the lungs (rate of cardiac output), CV is the venous concentration (entering the lungs). This equation is solved for CA.

For the liver compartment with mass input from blood and two outputs (venous blood and metabolism, without biliary excretion which in the case of PCE was negligible), the toxicant mass transfer is given by the equation:

$$dAL/dt = QL(CA - CVL) - dAM/dt$$

$$dAM/dt = (V_{MAX} \times CVL)/(Km + CVL) + KF \times CVL \times VL$$

$$CVL = AL/(VL \times PL)$$

where: AL is the amount in liver, QL is the blood flow through the liver, CA is the arterial concentration, CVL is the venous concentration, AM is the amount metabolized, $\times$ is the multiplication, V_{max} is pseudo-

maximal velocity rate of metabolism, Km is apparent Michaelis-Menten metabolism constant, KF is first order metabolism rate, VL is liver volume, and PL is liver/blood partition coefficient.

For the milk compartment with mass input from blood and two outputs (venous blood and milk), the toxicant mass transfer is given by the equation:

$$dAMAT/dt = QMT(CA - CVMT) - RPUP$$

$$RPUP = OUTX \times AMAT/V_{MILK}$$

where: AMAT is the amount in mammary tissue, QMT is the blood flow through the mammary tissue, CA is the arterial concentration, CVMT is the venous concentration (leaving the mammary tissue), RPUP is the rate of toxicant elimination with milk, OUTX = OUTI (zero order milk yield) during feeding, or OUTX = 0 during non feeding period, V_{milk} is volume of milk in mammary tissue.

For the infant's gastrointestinal tract compartment with mass input with milk and output with gastrointestinal absorption to blood, the toxicant mass transfer is given by the equation:

$$dMR/dt = RPUP - RAP$$

$$RAP = MR \times KAP$$

where: MR is the amount remaining in the gastrointestinal tract, RPUP is the rate of toxicant input with milk, RAP is the rate of absorption from gastrointestinal tract, $\times$ is multiplication, KAP is gastrointestinal absorption constant (fecal excretion has not been included as in the case of PCE it was negligible).

The daily dose of toxicant received with milk (IDM) is defined as the integral of RPUP over time divided by the number of days. Similarly, the total daily dose of toxicant (TOTINF) received by infant (with air and milk) is defined as the sum of IDM and IDI, the integral of the inhalation rate over given time divided by the number of days.

Extra cancer risk is defined as the product of the slope factor (SF), daily dose (IDM or TOTINF) and years of exposure (Y) divided by expected life span (70 years).

DISCUSSION

Examples and Program Validation

Several computer simulations of PCE concentrations in human blood and exhaled air were run with the milk compartment turned off, using exposure scenarios described in the literature (Stewart et al., 1970, 1961a,b; Fernandez et al., 1976; Bolanowska and Golacka, 1972). The set of physiological parameters used for model validation and simulations in humans is shown in Table 3.3-1. Results from the only documented case of the lactational transfer of PCE from mother to infant (Bagnell and Ellenberger, 1977) were also simulated with satisfactory fit of predicted PCE maternal blood and milk concentrations to the reported experimental data (Byczkowski and Fisher, 1993).

To test the program further, seven exposure scenarios for PCE were simulated using the measurements of PCE concentrations in indoor air made by the New York State Department of Health and published by Schreiber (1992). They determined PCE concentrations in dry-cleaning facilities, residential apartments, and apartments located just above the dry-cleaning facilities. It was assumed that a 7.2 kg infant ingests 0.7 L of breast milk per day for up to 1 year. The results of extra cancer risk assessment generated with our program are compared with those published by Schreiber (1992) in Table 3.3-2.

TABLE 3.3-1. KINETIC CONSTANTS AND PHYSIOLOGICAL PARAMETERS USED IN PBPK MODELING OF LACTATIONAL TRANSFER OF PCE IN HUMANS

Description	[Units]	Parameter	
Tissue Volumes	**[Fraction of Body Weight: BW]**		
Maternal			
Liver		VLC	= 0.04
Fat		VFC	= 0.2
Mammary		VMATC	= 0.05
Perinatal			
Infant Tissue		VTCP	= 0.9
	[L]		
Maternal			
Slowly Perfused		VS	= 0.79 × BW-VF-VMAT
Rapidly Perfused		VR	= 0.12 × BW-VL
Milk Volume		V_{MILK}	= 0.03542
Flow Rates	**[L/h/kg]**		
Maternal			
Alveolar Ventilation		QPC	= 19.7
Cardiac Output		QCC	= 18.0
Neonatal			
Alveolar Ventilation Infant		QPCP	= 25.2
Cardiac Output		QCCP	= 22.0
	[Fraction of Cardiac Output]		
Maternal			
Liver		QLC	= 0.25
Fat		QFC	= 0.05
Partition Coefficients	**[Ratio of Solubility]**		
Maternal			
Blood/Air		PB	= 19.8
Liver/blood		PL	= 6.83
Fat/Blood		PF	= 159.03
Slowly Perfused/Blood		PS	= 7.77
Rapidly Perfused/Blood		PR	= 6.83
Milk/Blood		P_{MILK}	= 2.8
Neonatal			
Blood/Air Infant		PPB	= 8.0
Other Tissues/Blood Infant		PPT	= 6.596
Metabolism			
Maternal			
	[mg/L]		
Apparent Michaelis-Menten		KM	= 0.32
	[mg/kg/h]		
Pseudo Maximal Velocity		V_{MAXC}	= 0.151

TABLE 3.3-2. THE ESTIMATED EXPOSURE OF INFANTS TO PCE IN BREAST MILK AND EXCESS CANCER RISK ASSESSMENT, ACCORDING TO BYCZKOWSKI AND FISHER (1994a)

Scenario ACC. to Schreiber (1992)	IDM [mg/kg/day]		ECRI	
	Schreiber (1992)	PBPK Model	Schreiber (1992)	PBPK Model
A	0.8200	0.3330	6.0×10^{-4}	2.4×10^{-4}
B	0.3400	0.1560	2.5×10^{-4}	1.1×10^{-4}
C	0.0800	0.0320	5.8×10^{-5}	2.3×10^{-5}
D	0.3000	0.2020	2.2×10^{-4}	1.5×10^{-4}
E	0.0500	0.0290	3.6×10^{-5}	2.1×10^{-5}
F	0.0015	0.0009	1.4×10^{-6}	6.6×10^{-7}
G	0.0001	0.0001	1.0×10^{-7}	7.3×10^{-8}

IDM - Daily infant dose of PCE from breast milk calculated by this program.

ECRI - Excess cancer risk from drinking PCE-contaminated breast milk.

Scenario: A - Occupationally-exposed mother inhaling air containing 50 ppm of PCE (340 mg/m^3), 8 h per day, five days per week, followed by exposure to an indoor residential background concentration of 0.0041 ppm (IRBC = 0.028 mg/m^3).

Scenario: B - Occupationally-exposed mother inhaling air containing 25 ppm of PCE (170 mg/m^3), 8 h per day, five days per week, followed by exposure to an IRBC of 0.0041 ppm.

Scenario: C - Occupationally exposed mother inhaling air containing 5.9 ppm of PCE (40 mg/m^3, approximate arithmetic mean concentration for counter-workers, pressers, and seamstresses), 8 h per day, five days per week, followed by exposure to an IRBC of 0.0041 ppm.

Scenario: D - Non-occupational exposed mother inhaling air containing 6.7 ppm of PCE (45.8 mg/m^3, the 24 h average concentration reported in an apartment located above a dry cleaner using an old dry-to-dry machine), seven days per week.

Scenario: E - Non-occupationally exposed mother inhaling air containing 1.1 ppm of PCE (7.7 mg/m^3, 24 h average concentration reported in apartments located above dry cleaners using transfer machines), seven days per week.

Scenario: F - Non-occupationally exposed mother inhaling air containing 0.037 ppm of PCE (0.25 mg/m^3, 24 h average reported above dry cleaners using dry-to-dry machines), seven days per week.

Secnario: G - Non-occupationally exposed mother inhaling air containing PCE at IRBC of 0.0041 ppm, seven days per week.

The resultant risk calculations from our simulations were moderately lower than values estimated by Schreiber (1992). Explanation of this apparent discrepancy became obvious when her estimated PCE concentrations in breast milk (CMAT) were compared with continuous simulations by our program (Figure 3.3-5a). Without the insight into the kinetics of mother's exposure (increasing during the 8 h workday, and decreasing after the work-shift and especially over the week-end), Schreiber (1992) assumed a maximum exposure, and thus, the peak PCE concentration in milk (CMAT) resulted in an overestimation of infant daily dose from milk (IDM; Figure 3.3-5b). Moreover, depending on the exposure scenario, the average daily dose may change with time, so it seems essential to follow the excess cancer risk in a time-dependent manner rather than to assume a static value. Despite the differences in assumptions, initial

conditions, and PBPK model construction, our exposure assessments come very close to those published by Schreiber (1992). Consequently, the excess cancer risk estimates calculated by our model for an infant breast-fed for 1 year, are quite close to the estimates presented by Schreiber (1992). It is suggested that modeling the toxicokinetics with our program gave more realistic approximations of PCE dose received by nursing infants than the estimates based on peak level dosemetrics. It seems that the exposure assessment by PBPK model may serve as a valuable tool in the overall risk assessment process for nursing infants.

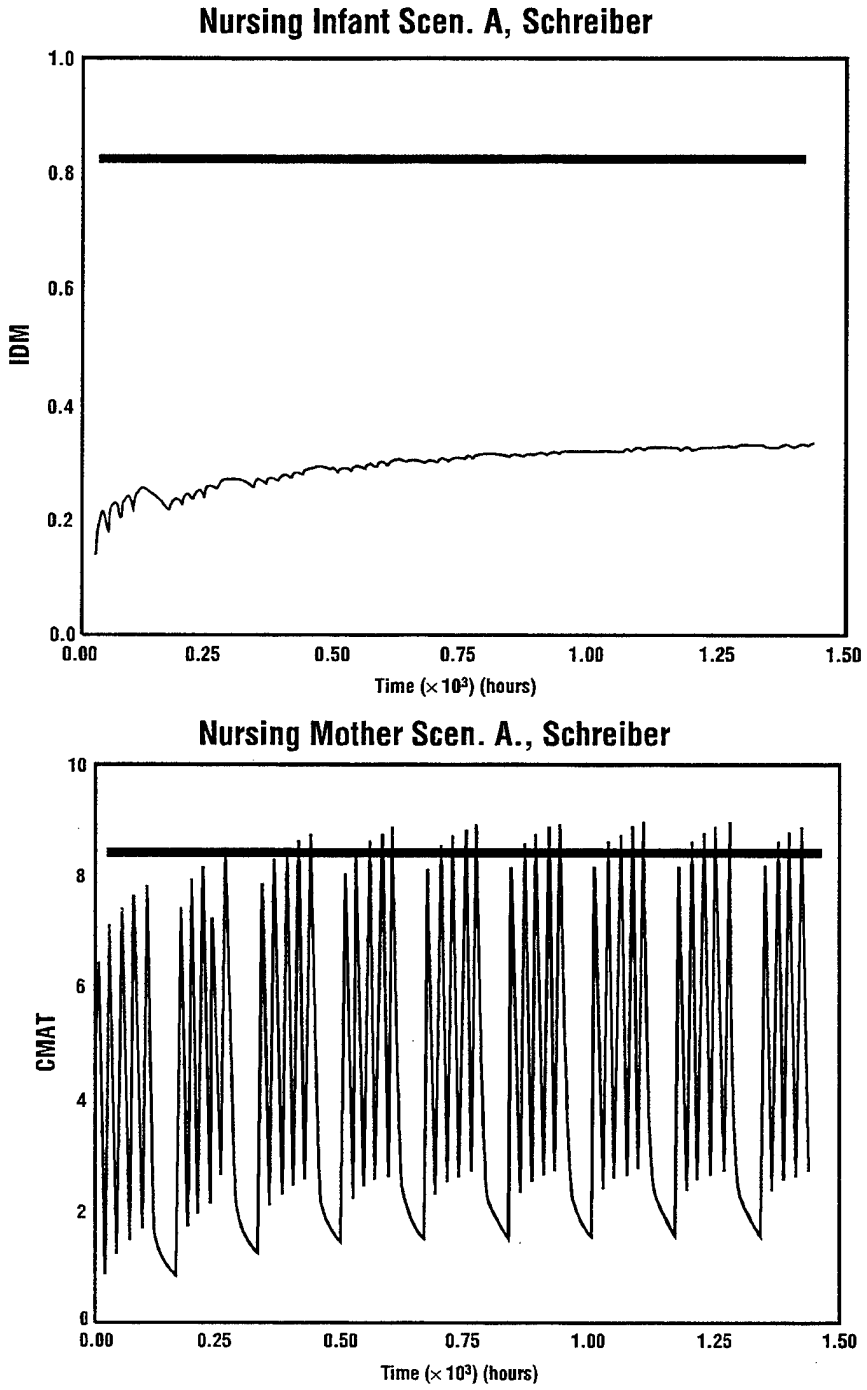

Figure 3.3-5. Comparison of Continuous PCE Toxicokinetics Simulation by PBPK Model (Thin Lines) with Values Estimated by Schreiber (1992) (Thick Lines). (a) Concentration of PCE in breast milk (CMAT) vs time [h]. (b) Infant daily dose of PCE received with milk (IDM) vs time [h]. The figures were originally created with SIMUSOLV software and redrawn for publication

REFERENCES

ACGIH. 1993. Threshold limit values for chemical substances and physical agents and biological exposure indices. pp. 28–38 (American Conference of Governmental Industrial Hygienists, Cincinnati, OH).

Bagnell, P.C. and H.A. Ellenberger. 1977. Obstructive jaundice due to a chlorinated hydrocarbon in breast milk. *Can. Med. Assoc. J.* 117:1047–1048.

Bolanowska W. and J. Golacka. 1972. Inhalation and excretion of tetrachloroethylene in men in experimental conditions. *Medycyna Pracy* 23:109–119.

Byczkowski, J.Z. and J.W. Fisher. 1993. Quantitative approach to assess risk from lactational transfer of tetrachloroethylene to breast-fed infants. Soc. Risk Analysis Annual Meeting, Program and Abstracts. pp. E-10 (Savannah, GA).

Byczkowski, J.Z. and J.W. Fisher. 1994a. Lactational transfer of tetrachloroethylene in rats. *Risk Analysis* 14:339–349.

Byczkowski, J.Z. and J.W. Fisher. 1994b. Tetrachloroethylene exposure assessment of breast-fed infants. *Toxicologist* 14:86(45).

Byczkowski, J.Z. and J.W. Fisher. In Press. A computer program linking physiologically based pharmacodynamic model with cancer risk assessment for breast-fed infants. *Comp. Meth. Progr. Biomed.*

Byczkowski, J.Z., E.R. Kinkead, H.F. Leahy, G.M. Randall, and J.W Fisher. 1994. Computer simulation of the lactational transfer of tetrachloroethylene in rats using a physiologically based model. *Toxicol. Appl. Pharmacol.* 125:228–236.

CDHS. 1991. Health effects of tetrachloroethylene (PCE). pp. 5-1–5-36 (Scientific Review Panel Version Technical Support Document Part B, State of California Air Resources Board, California Department of Health Services).

EPA. 1989. Risk assessment guidance for superfund. vol. 1, pp. 8-1–8-10. Document EPA/540/1-89/002 (U.S. Environmental Protection Agency, Washington, DC).

Fernandez, J., E. Guberan, and J. Caperos. 1976. Experimental human exposures to tetrachloroethylene vapor and elimination in breath after inhalation. *Amer. Ind. Hyg. Assoc. J.* 37:143–150.

Gargas, M.L., R.J. Burgess, D.E. Voisard, G.H. Cason and M.E. Andersen. 1989. Partition coefficients of low-molecular weight volatile chemicals in various liquids and tissues. *Toxicol. Appl. Pharmacol.* 98:87–99.

Gargas, M.L., M.E. Andersen, and H.J. Clewell III. 1986. A physiologically based simulation approach for determining metabolic constants from gas uptake data. *Toxicol. Appl. Pharmacol.* 86:341–352.

Mitchell and Gauthier Associates. 1987. Advanced Continuous Simulation Language (ACSL) Reference Manual (Concord, MA).

Ramsey, J.C., and M.E. Andersen. 1984. A physiologically based description of inhalation pharmacokinetics of styrene in rats and humans. *Toxicol. Appl. Pharmacol.* 73:159–175.

Schreiber, J.S. 1992. An assessment of tetrachloroethene in human breast milk. *J. Exp. Anal. Environ. Epidem.* 2:15–26.

Steiner, E.C., T.D. Rey, and P.S. McCroskey. 1990. Reference Guide Simusolv Modeling and Simulation Software (The Dow Chemical Co., Midland, MI).

Stewart, R.D., E.D. Baretta, H.C. Dodd, and T. Torkelson. 1970. Experimental human exposure to tetrachloroethylene. *Arch. Environ. Health* 20:224–229.

Stewart, R.D., D.S. Erley, A.W. Schaffer, and H.H. Gay. 1961a. Accidental vapor exposure to anesthetic concentrations of a solvent containing tetrachloroethylene. *Ind. Med. Surg.* 30:327–330.

Stewart, R.D., H.H. Gay, D.S. Erley, C. Hake, and A.W. Schaffer. 1961b. Human exposure to tetrachloroethylene vapor. *Arch. Environ. Health* 2:516–522.

4.1 PARTITION COEFFICIENTS AND GAS UPTAKE KINETICS OF HALON 1301 REPLACEMENT CANDIDATES, CF_3I, HFC-227ea, HFC-125, and FC-218

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ABSTRACT

Currently the Air Force uses Halon 1301 (trifluorobromomethane), a gaseous fluorocarbon, for aircraft fire fighting systems. CF_3I (trifluoroiodomethane), HFC-227ea (heptafluoropropane), HFC-125 (pentafluoroethane), and FC-218 (octafluoropropane) have been proposed as possible replacements for Halon 1301. Preliminary pharmacokinetics and metabolism of Halon 1301, CF_3I, HFC-227ea, HFC-215, and FC-218 were investigated. This was accomplished by exposing male Fischer-344 rats to Halon 1301, CF_3I, HFC-227ea, HFC-125, and FC-218 at starting chamber concentrations ranging from 100 to 5000 ppm in a closed system recirculating gas uptake chamber for a six-hour period. Samples were taken from the chamber and gas chromatography (GC) was used to determine the concentration in the chamber atmosphere. Data were used with a physiologically based pharmacokinetic (PBPK) model to estimate the metabolic constants K_{fc}(hr^{-1} kg^{-1}), K_m(mg L^{-1}) and V_{maxc}(mg hr^{-1} kg^{-1}). Halon 1301, HFC-227ea, and HFC-125 data were simulated without necessity of attributing any metabolic capacity by the rats. Simulation of CF_3I required some attribution of metabolic capacity. Simulation of FC-218 also required some attribution of metabolic capacity, although the amount of first order metabolism estimated was inconsistent due to the inability to detect any metabolites. The chemicals investigated generally showed a relatively low level of partitioning into tissues and were relatively inert metabolically.

INTRODUCTION

The purpose of this study was to measure the tissue-to-air partition coefficients and to describe the uptake kinetics of bromotrifluoromethane (Halon 1301) and its proposed replacement chemicals, iodotrifluoromethane (CF_3I), heptafluoropropane (HFC-227ea), pentafluoroethane (HFC-125), and octafluoropropane (FC-218), via closed chamber recirculating gas uptake methods.

Inhalation pharmacokinetics for all chemicals were determined experimentally in Fischer-344 (F-344) male rats. A physiologically based pharmacokinetic (PBPK) model was used to estimate the metabolism of the chemicals employing chemical-specific parameters and standard physiological constants.

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MATERIALS AND METHODS

Test Materials

CF_3I and Halon 1301 were obtained from PCR Inc., Gainesville, FL. HFC-227ea was obtained from Great Lakes Chemical Corp., West Lafayette, IN. FC-218 was obtained from 3M Inc., St. Paul, MN. HFC-125 was obtained from DuPont Chemicals Inc., Wilmington, DE. Mass spectral analysis revealed no impurities in any of the chemicals.

Animals

Male F-344 (200 to 350 g) rats (*Rattus norvegicus*) were obtained from Charles River Breeding Laboratories (Kingston, NY). Animals received Purina Formulab #5008 and softened water *ad libitum*. They were housed in plastic cages (2-3/cage) with hardwood chip bedding prior to exposure and were maintained on a 12-hour light / 12-hour dark light cycle at constant temperature (22 ± 1 °C) and humidity (40–60%). Cages were changed twice per week. Animals were marked for identification with a tail tattoo.

The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, National Research Council, DHHS. National Institute of Health Publication #86-23, 1985, and the Animal Welfare Act of 1966, as amended.

Partition Coefficients

Partition coefficients were determined by using a modified version of the vial-equilibration technique described by Gargas et al. (1989). Whole tissue was harvested and minced into a tissue slurry versus prepared as a tissue homogenate in saline. Rats used to determine partition coefficients were sacrificed with CO_2. Blood was collected from the posterior vena cava using a heparinized syringe. Liver, muscle (quadriceps), fat (epididymal and perirenal), and gastrointestinal (GI) tract (stomach and small intestine) were also removed for analysis. Blood samples (1.0 mL for all chemicals, except for FC-218, which was 2.0 mL) were placed in 12.4 mL glass vials and incubated/mixed for 3 h at 37 °C with 800 ppm of chemical in the vial head space. Whole tissue samples (1.0 g of liver and muscle, and 0.5 g of fat and GI for all chemicals, except FC-218, which was 2.0 g) were minced and incubated/mixed under the same condition as for blood, except fat was which equilibrated for 5–8 h. Partition coefficients were also determined at 80 and 400 ppm to show that they were concentration independent.

The chemical concentrations in the head space were analyzed using a HP19395A head space sampler (Hewlett-Packard, Avondale, PA) connected to a HP5890A gas chromatograph (GC) (Hewlett-Packard, Palo Alto, CA) equipped with a hydrogen flame ionization detector. Column selection and GC conditions varied for each chemical. For Halon 1301, HFC-227ea, HFC-125, and FC-218, a Chromopack PoraPLOT Q (Plot Fused Silica) 25m x 0.53 mm column was used. GC conditions were set with the detector temperature at 250 °C, injector temperature at 125 °C, helium carrier gas at 13.0 mL/min column flow, plus 13.0 mL/min make-up flow, and an oven temperature held constant at 70 °C for Halon 1301 and FC-218, 100 °C for HFC-227ea, and 75 °C for HFC-125. For CF_3I, a 12' × 1/8" stainless steel 10% SE-30, WHP 80/100 mesh Chromsorb column was used. Gas chromatography conditions were set with the detector temperature at 250 °C, injector temperature at 125 °C, nitrogen carrier gas flow at 30.0 mL/min, and an oven temperature held constant at 60 °C.

Metabolic Constants

A closed chamber recirculating gas uptake system with a volume of 8.0 L was used for the estimation of the whole animal metabolic constants (V_{maxc}(mg hr^{-1} kg^{-1}), K_m(mg L^{-1}), and/or K_{fc}(hr^{-1} kg^{-1})). Three F-344 rats were exposed to each study chemical using a gas uptake system similar to that described by Gargas et al. (1986). Initially, a predetermined concentration of the test chemical was introduced into the system so that the concentration in the chamber atmosphere would decrease as the chemical was taken up and metabolized by the rat. Four to five exposure concentrations were performed for 6 h for each chemical (Halon 1301 concentrations were 122, 1202, 2993, and 5557 ppm; CF_3I concentrations were 112, 648, 1228, 2727, and 5867 ppm; HFC-227ea concentrations were 112, 648, 1228, 2715, and 5867 ppm; HFC-125 concentrations were 132, 1005, 2725, and 5305 ppm; and FC-218 concentrations were 126, 1035, 1730, and 4825 ppm). Ascarite (75–150 g) was used as the CO_2 absorber. Oxygen concentrations were maintained at (± 1%) during the exposures. The system flow was maintained at 2.1 L/min with the flow to the sample loop of the GC at 100 mL/min.

The chemical concentrations in the chamber atmosphere were monitored every 5 min for the first 30 min and every 15 min thereafter using an automated gas sampling valve connected to a HP5890A GC. Chromatography was performed on a 25 m × 0.53 mm Chrompack PoraPLOT Q (Plot Fused Silica) column. The GC was equipped with a hydrogen flame ionization detector with a temperature of 250 °C, helium carrier flow at 12.1 mL/min with make-up flow of 14.2 mL/min, injector at 125 °C, and an oven temperature held constant at 80 °C for Halon 1301, at 125 °C for CF_3I, at 100 °C for HFC-227ea, at 70 °C for HFC-125, and at 70 °C for FC-218.

Model Development

Simusolv (DOW Chemical Co., Midland, MI), a FORTRAN-based continuous simulation language with optimization capabilities, was used on a VAX/VMS 8530 mainframe computer (Digital Equipment Corp., Maynard, MA). A PBPK model of the form described by Ramsey and Andersen (1984), with an additional compartment added to describe the GI tract, was used for estimating metabolic constants.

RESULTS

Partition Coefficients

Table 4.1-1 shows the rat tissue to air partition coefficients determined for Halon 1301, CF_3I, HFC-227ea, HFC-125, and FC-218 at 800 ppm, which were used in the PBPK model optimization. No difference was observed between partitions determined at 80, 400, or 800 ppm. Due to the extremely low partition coefficient for FC-218, 2.0 g or more of rat tissue were used.

TABLE 4.1-1 PARTITION COEFFICIENTS

(800ppm--Tissue to Air)

	Blood			Liver			Muscle			Fat			GI		
Chemical	Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.
1301	0.74	0.27	0.37	0.81	0.36	0.44	0.59	0.21	0.36	3.57	1.52	0.43	0.64	0.37	0.58
CF3I	1.73	0.28	0.16	1.27	0.21	0.17	1.32	0.18	0.14	10.35	0.82	0.08	1.61	0.38	0.24
FM 200	0.45	0.19	0.43	0.42	0.15	0.35	0.36	0.11	0.30	1.58	0.38	0.24	0.45	0.20	0.45
HFC-125	0.23	0.11	0.50	0.26	0.17	0.64	0.34	0.29	0.85	0.45	0.25	0.56	0.37	0.04	0.12
FC-218	0.25	0.13	0.55	0.07	0.09	1.35	0.18	0.09	0.54	0.04	0.12	2.86			

Tissue to Blood Ratios

Chemical	L:B	M:B	F:B	GI:B
1301	1.09	0.79	4.80	0.86
CF3I	0.73	0.77	6.00	0.94
FM 200	0.92	0.79	3.48	0.98
HFC-125	1.17	1.53	1.99	1.64
FC-218	0.29	0.71	0.16	

Gas Uptake Studies

Uptake of Halon 1301, HFC-227ea, and HFC-125 were simulated without the necessity of attributing any metabolic capacity by the rats. Simulation of uptake of CF_3I required some attribution of metabolic capacity by the rats. Distinction could not be made between attribution of both saturable (V_{maxc}(mg hr^{-1} kg^{-1}) = 0.375, K_m(mg L^{-1}) = 0.1) and first-order (K_{fc}(hr^{-1} kg^{-1}) = 1.6) metabolism with a chamber loss of 2.7% and attribution of saturable (V_{maxc}(mg hr^{-1} kg^{-1}) = 0.375, K_m(mg L^{-1}) = 0.1) metabolism alone with a chamber loss of 4%. This indicates a lack of discrimination between first order

metabolism and chamber loss for CF_3I. Simulation of uptake of FC-218 required some attribution of first-order metabolic capacity by the rats.

DISCUSSION

Halon 1301, HFC-227ea, and HFC-125 gas uptake data were simulated successfully by assuming that no metabolism of the chemical was occurring and that after initial uptake by the animal, further losses were those occurring in the uptake system itself. Simulation of the CF_3I and FC-218 required some attribution of metabolism by the rats beyond losses to the system. Another indication that the chemical CF_3I was disappearing beyond that taken up by the chamber is demonstrated by the chromatograms of the chamber air. As gas uptake experiments progressed, a second peak appeared and increased in size (data not shown). This could represent a metabolite resulting from the metabolism of the chemical by the rats or could represent a product resulting from spontaneous breakdown of CF_3I in the chamber. The peak appeared only when live rats were in the chamber with the presence of the parent chemical. However, further experiments would be necessary to determine the identity and origin of the second chromatographic peak. The need to attribute first-order metabolism to FC-218 is problematic as no metabolites could be detected and because of the expected inertness of a perfluorinated compound. Problems with the gas uptake methodology are still being investigated before any final conclusions can be reached about the metabolism of FC-218.

REFERENCES

Gargas, M.L., M.E. Andersen, and H.J. Clewell III. 1986. A physiologically based simulation approach for determining metabolic constants from gas uptake data. *Toxicol Appl. Pharmacol.* 86:341–352.

Gargas, M.L., R.J. Burgess, D.E. Voisard, G.H. Cason, and M.E. Andersen. 1989. Partition coefficients of low-molecular weight volatile chemicals in various liquids and tissues. *Toxicol. Appl. Pharmacol.* 98:87–99.

Ramsey, J.C. and M.E. Andersen. 1984. A physiologically based description of the inhalation pharmacokinetics of styrene in rats and humans. *Toxicol. Appl. Pharmacol.* 73:159–175.

4.2 ACUTE 15-MINUTE, NOSE-ONLY INHALATION EXPOSURES OF HALON 1301 TO MALE AND FEMALE SPRAGUE-DAWLEY RATS

E.R. Kinkead, M.L. Freedman, R.E. Wolfe, and H.F. Leahy

ABSTRACT

Acute 15-minute, nose-only inhalation exposures were performed on male and female Sprague-Dawley rats at 30%, 50%, and 80% Halon 1301. Slight tremors and ataxia were noted following 50% and 80% Halon 1301 exposures. All animals survived and gross examination at 14 days postexposure indicated normal findings. Results were compared with the potential Halon 1301 replacement candidate, trifluoroiodomethane.

INTRODUCTION

The flooding agent for extinguishing in-flight aircraft and electronic equipment fires currently being used by the Air Force is Halon 1301 (bromotrifluoromethane). Halon 1301 is a fully halogenated hydrocarbon of relatively low toxicity. However, environmental concerns about the contribution of halons to depletion of stratospheric ozone and global warming has resulted in an attempt to find adequate replacements. A compound being considered, because it is believed to have less ozone-depleting activity, is trifluoroiodomethane (CF_3I). Extensive testing is being performed on CF_3I, including short-term acute toxicity evaluations. Kinkead et al. (1994) reported nose-only inhalation exposures of CF_3I to male F-344 rats at concentrations of 0.5 and 1.0% in which all rats survived 4-hour exposures. Histopathologic examination of tissues at various time points following exposure indicated normal findings. Fifteen-minute, nose-only inhalation exposures have been performed on CF_3I at concentrations ranging between 13% and 29% (Skaggs et al., 1993; Ledbetter, 1994). A 15-minute inhalation median lethal concentration (LC_{50}) value for Sprague-Dawley rats is 27% CF_3I.

To make comparisons on short-term lethality of CF_3I and Halon 1301, it was necessary to perform 15-minute, nose-only inhalation exposures of Halon 1301 under conditions similar to those used to establish the toxicity of CF_3I. This will provide information necessary to make valid judgments in assessing the comparative risks of the two fire extinguishants.

MATERIALS AND METHODS

Test Compound

The Halon 1301, supplied from the Air Force inventory, is a liquid and was stored in a cylinder under pressure. Pertinent physical and chemical properties follow:

CAS No.	75-63-8
Systematic Name	Bromotrifluoromethane
Molecular Weight	148.91
Empirical Formula	$CBrF_3$
Physical State	Colorless gas
Vapor Density (Air = 1)	5.12
Melting Point	−168 °C
Boiling Point	−57.8 °C
Flash Point	Not flammable
Vapor Pressure	205 psi @ 21 °C
	(Approx. 10,600 mmHg)
Solubility in water	Very slight

Test Animals

Twenty male and twenty female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories, Raleigh, NC. The rats were 7 weeks of age upon arrival and 9 weeks of age at the time of exposure. All rats were identified by tail tattoo and were subjected to a 2-week quarantine period. Water and feed (Purina Formulab #5008) were available *ad libitum*, except during exposure. Animal room temperatures were maintained at 21–25 °C, and the light/dark cycle was set at 12-hour intervals. The animals were group housed (2 per cage, except during exposure) in clear plastic cages with wood chip bedding (Betta-Chip, Northeastern Products Corp., Warrensburg, NY).

Generation and Analysis of Exposure Atmospheres

Halon 1301 vapor was generated from a cylinder through a calibrated Matheson rotometer (Matheson Gas Products, Secaucus, NJ). The Halon 1301 vapor was combined with oxygen (20–21% of total flow) and nitrogen (N_2) which was added as required to produce a combined N_2 and 1301 mixture of 79–80%. The Halon 1301 concentration was continuously monitored using a flow-through, 10-cm path cell, with a Miran 1A infrared analyzer (Foxboro, S. Norwalk, CN) at the absorption band of 11.7 m. Calibration curves were developed using Tedlar sample bags (SKC, Eighty-four, PA). The flow-through chamber was monitored and the exposure concentration maintained prior to introducing the test animals. The animals were introduced to the exposure in a staggered fashion. Each rat was removed from the chamber and the port was resealed following 15 min exposure. Because the animals were introduced to the exposure system in a staggered fashion, the total chamber monitoring time was 20 min. The exposure concentrations were monitored continuously. A digital value was recorded at 5-minute intervals

and used for concentration calculation. Temperature and humidity within the Cannon chamber were not monitored. Oxygen concentration was monitored using a Hudson Oxygen Monitor (Model #5590, Hudson Electronics Division, Temeculda, CA), calibrated with room air as 21%.

The nose-only chamber was a stainless-steel flow past chamber as described by Cannon et al., 1983. The chamber had 52 ports; 10 were randomly selected for rat exposure. Plexiglas rat restraining tubes that extended radially outward were plugged into the ports. The remaining ports were capped or used for monitoring chamber atmosphere conditions.

Exposure Regimen

Each nose-only exposure was for 15 min. The flow-through exposure system was maintained at the target concentration prior to the introduction of the test animals. The containment tubes with animals were attached to the exposure system, one at a time, at 30-second intervals. The animals were withdrawn from the system in a similar manner. The exposure groups consisted of 5 male and 5 female rats per concentration level, and the rats were maintained 14 days postexposure for evaluation. A total of 15 male and 15 female rats were included in each of 3 exposures, at target concentrations of 30%, 50%, and 80% Halon 1301.

Toxicity Assessments

Records were maintained of body weights and signs of toxicity. Euthanasia was via halothane inhalation overdose. At sacrifice, gross pathology was performed on all rats.

RESULTS

Exposure System Analysis

The specified target concentrations of 30%, 50%, and 80% Halon 1301 were maintained during the 15-minute exposure periods. The exposure mean concentrations were maintained within ±2% of the desired concentrations. Mean concentrations for each exposure, along with the mean high and low concentrations, are provided in Table 4.2-1.

TABLE 4.2-1. ANALYSIS OF HALON 1301 CONCENTRATION INHALED BY MALE AND FEMALE SPRAGUE-DAWLEY RATS

Target Concentration (%)	30.0	50.0	80.0
Mean Concentration, N = 5 (%)	30.2	50.3	81.3
Standard Deviation	0.2	0.1	0.4
Maximum Concentration (%)	30.6	50.4	81.7
Minimum Concentration (%)	30.0	50.2	81.0

Inhalation Toxicity

There were no deaths resulting from exposure. Female rats exposed at 50% Halon 1301 showed mild tremors and slight incoordination following exposure. Both male and female rats demonstrated mild tremors and slight incoordination following the 80% exposure. The tremors and incoordination dissipated by 10-minute postexposure. Mean body weight gains during the 14-day observation period followed a normal pattern for Sprague-Dawley rats (Table 4.2-2) based on historical control data.

All rats were examined grossly at the conclusion of the 14-day observation period. No gross lesions that could be attributed to exposure were noted.

TABLE 4.2-2. BODY WEIGHTS[a] OF SPRAGUE-DAWLEY RATS EXPOSED FOR 15 MINUTES TO HALON 1301 VIA NOSE-ONLY INHALATION

	Day (pre-exposure and post-exposure)		
Halon 1301 Con. (%)	**0**	**7**	**14**
Males			
80	361.8 ± 19.6	398.6 ± 22.1	436.9 ± 22.7
50	343.2 ± 31.3	368.3 ± 46.9	411.4 ± 60.4
30	323.1 ± 6.1	368.6 ± 6.9	411.5 ± 9.3
Females			
80	256.7 ± 11.3	270.1 ± 16.5	290.5 ± 16.7
50	232.0 ± 12.5	261.4 ± 7.4	284.0 ± 15.6
30	223.7 ± 18.3	242.2 ± 17.2	263.9 ± 25.2

[a]Mean ± SD (N = 5)

DISCUSSION

Acute (15 min) inhalation of Halon 1301 at concentrations of 30%, 50%, and 80% (300,000, 500,000, and 800,000 ppm) did not result in mortality to either male or female Sprague-Dawley rats. Signs of toxic stress were limited to slight tremors and slight loss of coordination following exposure to 50% or 80% Halon 1301, which dissipated by 10-minute postexposure. Gross examination of tissues at 14 days postexposure indicated normal findings.

The lack of mortality in rats at these exposure concentrations is in agreement with that reported by Chambers and Krackow, 1950; Comstock et al., 1950; and MacNamee, 1950, in which rats survived exposures to high concentrations of Halon 1301 for 15 min. The lack of mortality in the rats exposed at these concentrations would indicate that Halon 1301 is less potent than CF_3I (15 min LC_{50} of 27%).

REFERENCES

Cannon, W.C., E.F. Blanton, and K.E. McDonald. 1983. The Flow-Past Chamber: An Improved Nose-Only Exposure System for Rodents. *Am. Ind. Hyg. Assoc. J.* 44(12):923–933.

Chambers, W.H. and E. H. Krackow. 1950. An investigation of the toxicity of fire extinguishing liquids. Part I. Summary. Medical Division Research Report No. 23, Chemical Corps, Army Chemical Center, MD.

Comstock, C.C., F.P. McGrath, S.B. Goldberg, and L.H. Lawson. 1950. An investigation of the toxicity of proposed fire extinguishing fluids. Part II. The approximate lethal concentration to rats by inhalation of vapors for 15 min. Medical Division Research Report No. 23, Chemical Corps, Army Chemical Center, Md.

Kinkead, E.R., S.A. Salins, R.E. Wolfe, H.F. Leahy, and J.H. English. In Press. Acute Toxicity Evaluation of Halon Replacement Trifluoroiodomethane (CF_3I). AL-TR-1994, Armstrong Laboratory, Wright-Patterson Air Force Base, OH.

Ledbetter, A. 1994. Personal communication.

MacNamee, J.K. 1950. An investigation of the toxicity of proposed fire extinguishing fluids. Part III. The pathology in rats produced by inhalation of proposed fire extinguishing fluids. Medical Division Research Report No. 23, Chemical Corps, Army Chemical Center, Md.

Skaggs, S.R., D.S. Dierdorf, and R.E. Tapscott. 1993. Update on Iodides as Fire Extinguishing Agents. International CFE and Halon Alternatives Conference, Washington, DC. pp. 800–808.

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4.3 CARDIAC SENSITIZATION POTENTIAL OF CF_3I (TRIFLUOROIODOMETHANE)

A. Vinegar and D.E. Dodd

ABSTRACT

CF_3I is being considered by the U.S. Air Force as a candidate for replacement of Halon 1301 (CF_3Br), the fire extinguishant currently used as a total flooding agent in both occupied and unoccupied spaces. As part of the preliminary screening of CF_3I, an initial evaluation of its toxicity, including cardiac sensitization potential, was undertaken. Cardiac sensitization testing of CF_3I using beagle dogs showed a no observable effect level at 0.2% and a lowest observable adverse effect level at 0.4%. Since the proposed use concentration for fire extinguishing is 7.0%, the cardiac sensitization results eliminate neat CF_3I for use as a flooding agent in occupied spaces, but it is still a viable option for use in unoccupied spaces.

INTRODUCTION

In response to the requirements of the Montreal Protocol of 1987, alternatives are being considered to ozone-depleting halons (used widely as fire-fighting agents). Halons, due to their chemical stability (contain bromine, chlorine, fluorine, and carbon), do not decompose in the lower atmosphere but instead migrate slowly into the stratosphere. When these compounds react with ultraviolet radiation, they release chlorine and bromine which then break apart the ozone molecule, leading to the stratospheric depletion of ozone. CF_3I has been considered by the military and Federal Aviation Administration as a replacement for Halon 1301 for use as a flooding agent in both occupied and non-occupied spaces.

Cardiac sensitization to adrenaline is a phenomenon associated with the inhalation of a number of unsubstituted and halogenated hydrocarbons (Beck et al., 1973; Clark and Tinston, 1973, 1982; Hardy et al., 1994). After inhalation of the sensitizing agent, challenge with adrenaline causes cardiac arrhythmias. The U.S. Environmental Protection Agency (EPA) has been mandated under Title VI of the U.S. Clean Air Act of 1990 (Public Law 101-549) to evaluate alternatives to Class I ozone depleting substances, including halon fire and explosion protection agents. Under the Significant New Alternatives Policy Program the EPA considers the cardiac sensitization potential as the most sensitive biological endpoint because of the potent sensitizing effect of these chemicals in the epinephrine-challenged dog model. Therefore, the cardiac sensitization test was performed on CF_3I.

METHODS

The Toxic Hazards Research Unit subcontracted the performance of this investigation with Huntingdon Research Centre Ltd. (HRC), Huntingdon, England. T.J. Kenny, C.K. Shepherd, and C.J. Hardy of HRC were the primary scientists involved in conducting the study. A brief presentation of the methods and results follow. Details of the methods and results are given in an Air Force technical report (In Press).

Test Compound

The CF_3I (CAS 2314-97-8) used in this study was purchased from PCR, Inc., Gainesville, FL. The compound has a formula weight of 195.91 and a boiling point of −22.5 °C. The compound is a liquid and is stored in a cylinder under pressure. Purity was confirmed by gas chromatography/mass spectrometry (GC/MS) analysis to be > 99.8%.

Test Animals, Accommodation and Husbandry

Nine purebred male beagle dogs (12.7–15.7 kg), approximately 6 to 7 months old, were used for the study. They were obtained from Interfauna UK Ltd, Abbots Ripton Rd., Wyton, Huntingdon, England. Fresh tap water and 400 g of dry diet (Diet A, Special Diets Services Ltd.) were provided daily. The dogs were acclimatized to laboratory conditions and handling procedures for approximately 4 weeks before experimental work began.

Exposure System

Dogs were exposed to the test gas by a snout-only system. A Halls face mask (Veterinary Drug Co.) was used. To ensure a reasonably airtight fit around the dog's snout, a latex sheet with a hole was placed over the snout end of the face mask, and the dog's snout protruded through the sheet into the mask. The test gas was metered from a pressurized cylinder through a flowmeter and added to a supply of air passing into the face mask. The relative flows of test gas and diluent air were adjusted individually to result in a total air flow of approximately 40 L/min at the mask. The test atmosphere was sampled continuously using a metal bellows pump and analyzed using a Miran 1A CVF infrared gas analyzer. The measuring wavelength for CF_3I was 8.55 μm for the concentration of range of 0.05-50% v/v in air. The analyzer was calibrated using gas sampling bags (SKC, Inc., USA) containing known concentrations of the test gas.

Measurement of the Electrocardiogram (ECG)

The standard lead II electrocardiogram was used throughout the study. Standard ECG limb leads were then connected to the prepared areas on the dog with blunt clips. The electrocardiograph (Devices 3442 ECG amplifier with a two-channel chart recorder) was calibrated with 1 mV peaks.

Experimental Procedure

The experimental procedure was based on published techniques (Reinhardt et al., 1971). Adrenaline solutions were prepared immediately prior to each exposure session from a stock solution (adrenaline 1:1000) and sterile pyrogen-free water. Injections were given at a rate of 0.1 mL/sec. Appropriate dilutions were used so that the volume of adrenaline solution used was consistently 0.1 mL/kg body weight.

Experimental Design

Stage 1: Each of the nine dogs on the study was tested with fresh air supplied to the face mask. Various concentrations of adrenaline solution were used in order to establish the response of each individual dog to adrenaline alone. An appropriate initial adrenaline dose was selected for each dog following review of the results of Stage 1 of the study.

Stage 2: Two dogs were selected for exposure to CFC to show that the experimental model used gave a positive response with a known cardiac sensitizer.

Stage 3: Each of the six dogs selected for exposures received air only for 17 min. At this point the adrenaline response of each dog was assessed and compared to previously reported responses. Each of the dogs selected was exposed to CF_3I according to the following schedule:

Exposure Session	Test Gas	Concentration in Air % v/v
1	Air only	
2	CF_3I	0.1
3	CF_3I	1.0
4	CF_3I	0.2
5	CF_3I	0.4

At least one calendar day was allowed between each exposure session to allow the dogs to recover. When a clear positive response was seen in any dog, no further exposures were undertaken on that animal. If a dog died as a result of exposure to the test gas, no further exposures at that concentration or higher concentrations were performed.

Interpretation of Results

The study was designed to provide information on any dose level that would give rise to clear signs of test gas-related cardiac sensitization. The criterion for a positive effect was the appearance of a burst of multifocal ventricular ectopic activity or ventricular fibrillation. Ventricular tachycardia alone was not necessarily considered definitive evidence of a positive response.

RESULTS AND DISCUSSION

Stage 1 — Challenges with Adrenaline Alone

The response of a dog to administration of adrenaline was dependent on the dose given and the responsiveness of each individual animal. Typically, the response consisted of a transient increase in heart rate followed by a slowing of heart rate and an increase in the height of the T-wave. In some dogs multiple unifocal ventricular tachycardia occurred. On the basis of the results, the adrenaline dose selected for the dogs was 1, 4, 8, or 12 μg/kg.

Stage 2 — Exposure to CFC

Two dogs were selected for Stage 2. The first dog produced a positive response at 2% in air with fatal ventricular fibrillation. The other was not exposed due to humane considerations. These data are consistent with previously published data (Reinhardt et al., 1971) and show that the test is capable of detecting substances that are cardiac sensitizers.

Stage 3 — Exposure to CF_3I

All dogs were tested at 0.1% CF_3I in air. All dogs were negative and nothing abnormal was observed. One dog (1147) was tested at 1.0% CF_3I in air. The dog responded positively and died. All remaining dogs were tested at 0.2% CF_3I in air. All dogs were negative and nothing abnormal was observed. One dog (1149) was tested at 0.4% CF_3I in air. The dog responded positively and died. Exposures were terminated. A summary of cardiac responses to adrenaline administration during CF_3I is shown in Table 4.3-1.

TABLE 4.3-1. SUMMARY OF CARDIAC RESPONSES TO ADRENALINE ADMINISTRATION DURING CF_3I EXPOSURE

	Adrenaline Dose	Concentration in Air			
Dog Number	(μg/kg)	0.1%	0.2%	0.4%	1.0%
1147	8	(N)	NP	NP	FVF(P)
1149	8	(N)	(N)	FVF(P)	NP
1153	8	(N)	(N)	NP	NP
1155	1	(N)	(N)	NP	NP
1157	4	(N)	(N)	NP	NP
1159	1	(N)	(N)	NP	NP
Incidence of positive response		0/6	0/5	1/1	1/1
Cumulative % positive response		0	0	100	100

FVF = Fatal ventricular fibrillation.
NP = Not performed.
(N) = Negative response.
(P) = Positive response.

SUMMARY

Cardiac sensitization testing showed a no observable effect level at 0.2% and a lowest observable adverse effect level at 0.4% CF_3I.

REFERENCES

Beck, P.S., D.G. Clark, and D.J. Tinston. 1973. The pharmacological actions of bromochlorodifluoromethane (BCF). *Toxicol. Appl. Pharmacol.* 24:20–29.

Clark, D.G. and D.J. Tinston. 1973. Correlation of the cardiac sensitisation potential of some halogenated and non-halogenated hydrocarbons. *Brit. J. Pharmacol.* 49:355–367.

Clark, D.G. and D.J. Tinston. 1982. Acute inhalation toxicity of some halogenated and non-halogenated hydrocarbons. *Human Toxicol.* 1:239–247.

Hardy, C.J., P.C. Kieran, and I.J. Sharman. 1994. Assessment of the cardiac sensitization potential (CSP) of a range of halogenated alkanes. *Toxicologist*, 14:378.

Reinhardt, C.F., A. Azar, M.E. Maxfield, P.E. Smith, Jr., and L.S. Mullin. 1971. Cardiac arrhythmias and aerosol "sniffing". *Arch. Environ. Health*, 22:265–279.

4.4 RESULTS OF GENOTOXICITY TESTING ON TRIFLUOROIODOMETHANE (CF_3I) AND 1,1,1,2,3,3,3-HEPTAFLUOROPROPANE (HFC-227ea)

D.E. Dodd and A. Vinegar

ABSTRACT

CF_3I and HFC-227ea are being considered by the U.S. Air Force as candidates for replacement of Halon 1301 (CF_3Br), the fire extinguishant currently used as a total flooding agent in both occupied and unoccupied spaces. As part of the preliminary screening of CF_3I and HFC-227ea, an initial evaluation of their genotoxicity was undertaken. CF_3I was positive in the Ames *Salmonella typhimurium* histidine reversion mutagenesis assay. The L5178Y/*tk* mouse lymphoma cell mutagenesis assay showed that CF_3I did not induce gene or chromosomal mutations in mammalian cells *in vitro*. A positive evaluation in the mouse bone marrow micronucleus test indicated CF_3I was clastogenic *in vivo*. For HFC-227ea, negative results were obtained from the Ames assay, the mouse micronucleus test, and the mouse lymphoma cell assay.

INTRODUCTION

In response to the requirements of the Montreal Protocol of 1987 alternatives are being considered to ozone-depleting halons (used widely as fire-fighting agents). Halons, due to their chemical stability(contain bromine, chlorine, fluorine, and carbon), do not decompose in the lower atmosphere but instead migrate slowly into the stratosphere. When these compounds react with ultraviolet radiation, they release chlorine and bromine which then break apart the ozone molecule, leading to the stratospheric depletion of ozone. CF_3I and HFC-227 ea are being considered by the military and the Federal Aviation Administration as potential replacements for Halon 1301 as a flooding agent in both occupied and non-occupied spaces.

An initial battery of genotoxicity tests were performed to evaluate the potential for tumorogenic activity of CF_3I and HFC-227ea. This battery included the Ames *Salmonella typhimurium* histidine reversion assay, the L5178Y/$tk^{+/-}$ mouse lymphoma cell mutagenesis assay, and the *in vivo* mouse bone marrow erythrocyte micronucleus test.

METHODS

The Toxic Hazards Research Unit subcontracted the performance of these investigations with Genesys Research, Inc., Research Triangle Park, NC. Dr. A. Mitchell of Genesys was the Study Director for these studies. A brief presentation of the methods and results follow. Details of the methods and results are given in six Air Force technical reports (AL/OE-TR-1995-0008, Vol. I, II, and III, and AL/OE-TR-1995-0009, Vol. I, II, and III).

Test Compound

The CF_3I (CAS 2314-97-8) used in this study was purchased from PCR, Inc., Gainesville, FL. The compound has a formula weight of 195.91 and a boiling point of −22.5 °C. The compound is a liquid and is stored in a cylinder under pressure. Purity was confirmed by Gas Chromatography/Mass Spectrometry (GC/MS) analysis to be > 99.8%. The HFC-227ea (CAS 431-89-0) was received from Great Lakes Chemical Corporation in a steel gas container. HFC-227ea has a formula weight of 170 and is a colorless gas. Purity was confirmed by GC/MS analysis to be 99.0%.

General Procedures

Testing was conducted in accordance with the provisions of the United States Environmental Protection Agency/Toxic Substances Control Act Good Laboratory Practice (GLP) Standards as defined in the Federal Register, August 17, 1989 (40 CFR, Part 792), TSCA Test Guidelines, Federal Register, September 27, 1985 (Vol. 50, #188, Part 798.5265, 798.5300, and 798.5395), and its revision (May 20, 1987, Vol. 52, #97). CF_3I and HFC-227ea concentrations were determined by infrared (IR) which was calibrated using a "closed-loop" method. The IR settings were: wavelength, 9.05 or 9.7 μ; absorbance, 0.25; and range, X1 for all genotoxicity studies. Pathlength ranged from 6.75 to 15.75 m and slit ranged from 1 to 2, dependent on the conditions of the study. Analyses were performed by withdrawing a test atmosphere sample into a gas-tight syringe and injecting it into the IR instrument.

***SALMONELLA TYPHIMURIUM* HISTIDINE REVERSION ASSAY**

Tester Strains

The tester strains used in this study were TA1535, TA1537, TA1538, TA98, and TA100, obtained from Dr. Bruce Ames of the University of California at Berkeley (Ames et al., 1975).

Method

The specific test method has been described in detail (Ames et al., 1975; Maron and Ames, 1983). After the top agar had set, the test material (or negative control) plates for all five strains that were to be exposed to one concentration of the test material (or to air, the negative control) were placed on a shelf on the bottom section of a plastic exposure chamber (Modular Incubator

Chamber, Billups-Rothenberg, Del Mar, CA) with an internal volume of approximately 1 L. The chambers, which consisted of an upper and lower section connected by a gasket and a large stainless steel adjustable squeeze clamp, were then closed by connecting and sealing the upper and lower sections.

Exposure Method

The test material and dilution air were metered through calibrated flowmeters into the exposure chambers, and chamber atmosphere samples were collected, via a gas-tight syringe, and injected into the IR instrument for analysis. The test material and the dilution air were adjusted until the desired chamber concentration was obtained. The chamber exhaust was first disconnected, and then the gases were shut off. This was done to prevent diluting the chamber atmosphere. The inlet and exhaust ports were then sealed with screw clamps. During the mutagenesis assay, two chambers per exposure level were required to hold all the petri plates. To ensure that both chambers received the same concentration, the gas was introduced to the inlet port of one chamber which was exhausted into the inlet of the second chamber. The exhaust from the second chamber was then analyzed by IR.

L5178Y/tk$^{+/-}$ MOUSE LYMPHOMA CELL MUTAGENESIS ASSAY

Cell Culture

L5178Y mouse lymphoma cells, clone 3.7.2C, provided by Dr. Donald Clive, Burroughs Wellcome Co., Research Triangle Park, NC, were stored in liquid nitrogen. The cells were grown as a suspension culture in F_{10HP} medium (see composition below), cleansed of homozygous ($tk^{-/-}$) cells with medium containing 0.1 μg/mL methotrexate, as described by Mitchell et al., 1988, and used as target cells for chemical exposure.

Exposure of Cell Cultures

For testing these volatile materials in the preliminary concentration range-finding and mutagenesis assays, three sterile 15 mL round-bottom glass blood tubes, sealed with red rubber serum stoppers, were prepared for each concentration level: a tube for the culture tested without activation, a tube for the culture tested with activation, and a sham tube that contained medium only (no cells or S9) which was used to estimate postexposure infrared (IR) analysis of the concentration of test material in the other two tubes.

MOUSE BONE MARROW MICRONUCLEUS TEST

Animal Source, Environment, Husbandry, and Evaluation

Thirty male and 30 female Swiss Webster mice, approximately 42 days old at receipt, were purchased from Charles River Laboratories in Raleigh, NC.

Administration of the Test Materials

Eight groups (group size = 5/sex) of Swiss Webster mice were exposed 6 h per day for 3 consecutive days at concentrations of either 0% (air-only control), 2.5%, 5.0%, or 7.5% CF_3I or 0%, 2.6%, 5.3%, or 10.5% HFC-227ea via nose-only inhalation. Additional groups served as positive controls and were dosed on exposure Day 3 via ip injection. The CF_3I or HFC-227ea mice were exposed in Cannon 52-port nose only chambers (Lab Products, Maywood, NJ), and the negative control mice were exposed in a nose-only chamber made by IN-TOX Products (Albuquerque, NM). For each exposure group, the test atmosphere was generated by metering the CF_3I or HFC-227ea gas from the cylinder into either a 2000 or 4000 mL Erlenmeyer flask that served as a mixing plenum. Oxygen levels from the chamber exhausts were determined using an O_2/Explosion meter (MSA Model 421).

Positive Control

Approximately 24 h prior to sacrifice positive control animals received one ip dose of 0.4 mg/kg triethylenemelamine.

Sacrifice and Slide Preparation

Slides of peripheral blood smears were made for all animals at 24 ± 3 h after the last exposure by the following procedure.

Each mouse was sacrificed by cervical dislocation and 2–3 µL of blood was obtained from the tail vein. The blood was mixed with 2–3 µL of calf serum, spread thinly on a slide and allowed to dry. Three slides were prepared per mouse. Slides were fixed in methanol, allowed to dry, stained with 5% Giemsa, rinsed and dried. Slides were divided into three sets, coded and inspected (without knowledge of the code) by two separate observers (one set/observer). Slides were first observed under low microscopic power, then scored at 100 × magnification (oil immersion). Cells contained more than one micronucleus were scored as a single micronucleated cell.

Raw Data Collection and Analysis

After all microscopic analysis was completed, the slides were again decoded, and the slide numbers, the ratios of PCEs per 2000 erythrocytes (CF_3I) or 1000 erythrocytes (HFC-227ea), and the number of micronuclei observed in approximately 1000 PCEs (CF_3I) or 200 PCEs (HFC-227ea) per mouse were recorded for each experimental and control animal. Because five mice per sex were exposed per treatment group, the PCE ratios for each group were calculated for 10,000 erythrocytes (CF_3I) or 5000 erythrocytes (HFC-227ea) per group, and the average number of micronuclei was calculated for 5000 PCEs (CF_3I) or 1000 PCEs (HFC-227ea) per group.

RESULTS AND DISCUSSION

***Salmonella Typhimurium* Histidine Reversion Assay**

Concentrations tested in the mutagenesis assay of CF_3I (Table 4.4-1) ranged from 1060 to 85,908 ppm CF_3I. Toxicity as indicated by a slight reduction in the background lawn (SR) was noted only for strain TA98 in the absence of metabolic activation, but toxicity as indicated by a decrease in the number of colonies was observed at the highest concentration tested, 85,908 ppm CF_3I, for strain TA1537 in the presence of activation, and strains TA1535 and TA100 in the absence and presence of activation. Concentrations tested in the Ames assay of HFC-227ea ranged from 438,707 to 934,548 ppm HFC-227ea. Toxicity was observed at the highest concentration for all five strains in the absence and presence of activation (data not shown). There was no evidence of a mutagenic response in any strain without or with activation for HFC-227ea.

As summarized in Table 4.4-1, CF_3I induced a positive mutagenic response as indicated by a concentration-related increase in mean histidine revertant colonies/plate, in the absence and presence of activation, in four of the five tester strains. Of the three strains that detect frameshift mutations, TA1538 was negative without and with activation; TA1537 yielded a positive response at ~5X background (the negative control) without activation and ~3X background with activation; and TA98 yielded positive responses that were ~4X and ~3X background without and with activation, respectively. More pronounced positive responses were obtained in the two strains that detect base-pair substitution mutations. In strain TA1535, revertant colonies were ~60X background without and with activation, with both responses higher than the respective positive controls. In strain TA100, the number of revertant colonies was ~12 background without activation and ~8.5X background with activation. Thus, without and with activation, CF_3I was mutagenic inducing frameshift and, particularly, base-pair mutations in *Salmonella typhimurium*.

TABLE 4.4-1. *SALMONELLA TYPHIMURIUM* MUTAGENESIS ASSAY OF IODOTRIFLUOROMETHANE (CF_3I)

Chemical	S9	Dose Per Plate	TA1535 Histidine Revertant Colonies/Plate		TA1537 Histidine Revertant Colonies/Plate		TA1538 Histidine Revertant Colonies/Plate		TA98 Histidine Revertant Colonies/Plate		TA100 Histidine Revertant Colonies/Plate	
			Mean ± S.D.	Notes	Mean ± S.D.	Notes	Mean ± S.D.	Notes	Mean ± S.D.	Notes	Mean ± S.D.	Notes
Air	–	N/A	23 ± 3.0		10 ± 3.7		22 ± 4.6		22 ± 5.2		96 ± 10.	
CF_3I	–	1,060 ppm	80 ± 12.1	*	9 ± 4.0		21 ± 2.5		28 ± 8.6	SR	130 ± 16.	
	–	2,775 ppm	173 ± 14.0	*	14 ± 4.5		30 ± 6.5		31 ± 4.5	SR	234 ± 64.	*
	–	10,586 ppm	522 ± 123.4	*	21 ± 3.2		17 ± 5.0		53 ± 6.7	SR,*	509 ± 43.	*
	–	23,230 ppm	1,395 ± 97.1	*	32 ± 5.9	u	35 ± 1.2		88 ± 17.3	SR,u	1,135 ± 89.	*
	–	85,908 ppm	341 ± 96.1	4+,*	49 ± 12.5	4+,*	20 ± 5.6	4+	88 ± 31.5	4+,*	549 ± 97.	4+,*
Sodium Azide	–	1.5 μg	577 ± 18.9	*							1,105 ± 178.	*
9-AA	–				682 ± 352.3	*						
4-NOPD	–						241 ± 5.5	*	211 ± 21.1	*		
Air	+	N/A	16 ± 4.6		9 ± 3.9		26 ± 5.8		31 ± 2.4		111 ± 6.	
CF_3I	+	1,060 ppm	41 ± 16.0		13 ± 3.5		16 ± 3.8		38 ± 16.1		130 ± 6.	
	+	2,775 ppm	119 ± 30.3	*	10 ± 4.7		15 ± 4.2		38 ± 1.7		237 ± 11.	*
	+	10,586 ppm	267 ± 11.2	*	16 ± 4.7		29 ± 4.2		58 ± 1.5		488 ± 57.	*
	+	23,230 ppm	915 ± 305.0	*	30 ± 5.5	*	22 ± 1.0		92 ± 15.5	u	936 ± 106.	*
	+	85,908 ppm	501 ± 93.1	4+,*	22 ± 3.5	4+	33 ± 3.6	4+	100 ± 39.1	4+,*	797 ± 43.	4+,*
2-Anthramine	+	2.5 μg	276 ± 62.4	*	162 ± 32.7	*	930 ± 144.6	*	1,035. ± 116.6	u	1,381 ± 88.	*

4-NOPD = 4-Nitro-*o*-phenylenediamine
9-AA = 9-Aminoacridine
* = $p < 0.05$
4+ = Normal background lawn
SR = Slight reduction in background lawn

L5178Y/*tk*$^{+/-}$ Mouse Lymphoma Cell Mutagenesis Assay

It may be noted that although the provided material safety data sheet for CF_3I indicated that this test material is not soluble in water, apparently CF_3I is at least partially soluble in medium, as the concentration-related depressions in relative suspension growth that were observed in the preliminary assay (data not shown) would not be expected with an insoluble material. For HFC-227ea, there was no toxicity in either the range-finding or mutagenesis assay (data not shown). Nominal concentrations of HFC-227ea ranged from 100,000 to 900,000 ppm. Thus, HFC-227ea may have been insoluble in cell culture medium. In the mutagenesis assay, HFC-227ea did not induce gene or chromosomal mutations (data not shown).

In the mutagenesis assay of CF_3I (Table 4.4-2), the average absolute cloning efficiencies of the negative controls (air) were 127.3% in the absence of activation and 136.1% in the presence of metabolic activation (data not shown); the spontaneous mutation frequencies averaged 57×10^{-6} in the absence of activation and 62×10^{-6} in the presence of activation. Therefore, both the cloning efficiencies and the spontaneous mutation frequencies met the criteria for acceptability. Positive control mutant frequencies were within the historical ranges for the laboratory. For both positive controls, primarily small (s) colony mutants were produced.

TABLE 4.4-2. RESULTS FROM THE L5178Y/*tk*$^{+/-}$ MOUSE LYMPHOMA MAMMALIAN CELL MUTAGENESIS ASSAY OF IODOTRIFLUOROMETHANE (CF_3I) IN THE ABSENCE AND PRESENCE OF METABOLIC ACTIVATION

Chemical	+/–S9	Conc.*	RTG (%)	MF × 10^{-6}	IMF × 10^{-6}	Notes
Air	–	N/A	114.39	54		
Air	–	N/A	86.31	60		
CF_3I	–	125,000 ppm	80.46	58	1	
CF_3I	–	250,000 ppm	79.93	55	—	
CF_3I	–	500,000 ppm	55.42	51	—	
CF_3I	–	650,000 ppm	49.20	76	19	
CF_3I	–	750,000 ppm	42.42	71	14	
CF_3I	–	900,000 ppm	22.61	75	18	
CF_3I	–	1,000,000 ppm	22.41	78	21	
Hycanthone	–	7.50 μg/mL	21.15	448	390	uu
Hycanthone	–	10.00 μg/mL	14.87	791	734	uu
Air	+	N/A	102.84	54		
Air	+	N/A	95.86	70		
CF_3I	+	125,000 ppm	96.77	60	—	
CF_3I	+	250,000 ppm	106.64	—**	—**	—**
CF_3I	+	500,000 ppm	81.60	63	1	
CF_3I	+	650,000 ppm	64.90	76	14	
CF_3I	+	750,000 ppm	60.34	74	12	
CF_3I	+	900,000 ppm	37.67	73	11	
CF_3I	+	1,000,000 ppm	28.28	67	5	
Cyclophosphamide	+	1.00 μg/mL	50.12	374	312	uu
Cyclophosphamide	+	2.00 μg/mL	10.63	896	834	uu
Cyclophosphamide	+	3.00 μg/mL	2.56	2,303	2,241	uu

* = Nominal Concentration.
RTG(%) = Percent relative total growth.
MF = mutant frequency.
IMF = induced mutant frequency.
uu = IMF $\geq 100 \times 10^{-6}$.
**Not cloned for mutagenesis.

As illustrated in Table 4.4-2, induced mutation frequencies of only 14 to 21 $\times 10^{-6}$ were obtained for the 4 highest tested concentrations in the absence of activation, and only 1 to 14 $\times 10^{-6}$ for the 5 highest tested concentrations in the presence of activation, and these increases were not concentration-related. Therefore, there was no evidence to suggest that induced mutation frequencies of at least 70 $\times 10^{-6}$ (required for evaluating the result as positive, +) would be obtained with greater toxicity, e.g., in the range of 10 to 20% RTG. For this reason, and considering biological significance, the results obtained for CF_3I in the L5178Y/*tk*$^{+/-}$ mouse

lymphoma cell mutagenesis assay in the absence and presence of activation are evaluated as negative (–). Therefore, when tested to the maximum concentrations that could be obtained under the conditions of testing, iodotrifluoromethane (CF_3I) did not induce gene or chromosomal mutations in mammalian cells *in vitro*.

Mouse Bone Marrow Micronucleus Test

No mice died during the CF_3I or HFC-227ea studies. All mice appeared normal throughout the studies. When weighed before exposure to CF_3I in the micronucleus assay, male mice weighed 27.4 to 33.0 g and female mice weighed 24.0 to 28.8 g. When weighed after exposure and immediately before sacrifice, male mice weighed 23.5 to 31.0 g and female mice weighed 19.9 to 27.2 g. The majority of the mice in all exposure groups, including the negative control group, lost weight from Day 1 (randomization) to Day 4 (sacrifice). Weight loss averaged 2.3 g for the male mice and 2.1 g for the female mice, and the 5.0% and 7.5% animals lost more weight than the negative controls and 2.5% animals. Thus, in addition to effects attributable to the mice being in the nose-only tubes for approximately 7 h per day, CF_3I-related weight loss was observed. For HFC-227ea, weight loss was also observed, but it was not test-material related.

Summaries of the results obtained in the micronucleus assay of CF_3I in male and female mice are presented in Table 4.4-3. Negative control values were within historical ranges, and each positive control group yielded a positive response that was significant at $p < 0.01$. The key indicators for this assay are the number of newly formed PCEs per 1000 erythrocytes, which is a measure of toxicity, and the number of MN per 1000 PCEs, which is an index of potential chromosome breakage.

In the negative control animals, PCE ratios were 11.1‰ for male mice and 20.8‰ for female mice. Both ratios were within historical control ranges for the laboratory, and no physiological basis for the difference in ratios between the sexes was apparent. Appropriately low numbers of MN/1000 PCEs were observed in the negative control mice: 2.0‰ in male mice and 1.0‰ in female mice, values which were also within historical ranges for the laboratory.

TABLE 4.4-3. MICRONUCLEUS ASSAY OF IODOTRIFLUOROMETHANE (CF_3I) IN MICE

Chemical	Average Dose	Average per Dose	
		PCEs/1000 Erythrocytes	MN/1000 PCEs
Males			
Air	N/A	11.1	2.0
CF_3I	2.5%	12.8	3.4
CF_3I	5.0%	6.7	5.2*
CF_3I	7.5%	8.7	9.0**
TEM	0.4 mg/kg	2.8**	24.6**
Females			
Air	N/A	20.8	1.0
CF_3I	2.5%	7.3**	2.4
CF_3I	5.0%	5.3**	3.0*
CF_3I	7.5%	4.9**	4.8**
TEM	0.4 mg/kg	3.3**	34.1**

MN = Micronuclei.
PCE = Polychromatic erythrocytes.
TEM = Triethylenemelamine.
* = $p < 0.05$.
** = $p < 0.01$.

PCE/erythrocyte ratios were depressed and MN/PCE ratios were elevated in the positive control mice. The PCE ratios had normal residuals and equal variances among all potential effects (dose, sex, and dose by sex), and the sex of the animals influenced the effect of the positive control ($p = 0.0290$). The MN ratios did not have normal residuals or equal variances; therefore, the natural log transformation was used on MN ratios. The natural logs of MN ratios had normal residuals and equal variances. The interaction between the dose of TEM and the sex of the animals was not statistically significant ($p = 0.0541$), but the effect of TEM dose was statistically significant ($p = 0.01$) for both sexes, as indicated in Table 4.4-3.

Toxicity of CF_3I was evidenced by dose-related depressions in weight for both sexes, statistically significant dose-related depressions in PCE ratios for female mice, and apparently biologically relevant depressions in PCE ratios for all except one male mouse. Positive responses, as

indicated by dose-related increases in micronuclei were observed in erythrocytes from both male and female mice, results which indicate that CF_3I is capable of inducing structural chromosomal aberrations *in vivo*.

For HFC-227ea, neither criterion for a positive response - a dose-related increase in micronuclei and the induction by one or more of the doses of a statistically significant ($p < 0.5$) increase in micronuclei induction - were observed in the male or female mice (data not shown). Therefore, HFC-227ea was evaluated as negative in the mouse bone marrow micronucleus test. Hence, HFC-227ea was not clastogenic *in vivo*.

SUMMARY

- CF_3I was positive in the *Salmonella typhimurium* histidine reversion mutagenesis assay. HFC-227ea was negative in the Ames assay.
- The L5178Y/*tk* mouse lymphoma cell mutagenesis assay showed that neither CF_3I nor HFC-227ea induced gene or chromosomal mutations in mammalian cells *in vitro*.
- Positive results in the mouse bone marrow micronucleus test indicated CF_3I was clastogenic *in vivo*. HFC-227ea was not clastogenic *in vivo*.

The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, Department of Health and Human Services, National Institute of Health Publication #86-23, 1985, and the Animal Welfare Act of 1966, as amended.

REFERENCES

Ames, B.N., J. McCann, and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test. *Mutat. Res.* 31:47–364.

Maron, D.M. and B.N. Ames. 1983. Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.* 113:173–215.

Mitchell, A. D., B. C. Myhr, C. J. Rudd, W. J. Caspary and V. C. Dunkel. 1988. Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: Methods used and chemicals evaluated. *Environ. Molec. Mutagen.* 12, Suppl. 13:1–18.

4.5 TWO-WEEK RANGE-FINDING, NOSE-ONLY INHALATION EXPOSURE OF F-344 MALE RATS TO TRIFLUOROIODOMETHANE (CF_3I)

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ABSTRACT

Trifluoroiodomethane (CF_3I) is being considered as a replacement compound for Halon 1301. The material would be used as a flooding agent for in-flight aircraft and electronic equipment fires and for fire extinguishment in confined spaces. As part of the process to develop environmental and health effects criteria, inhalation exposure during routine or repeated use conditions was examined. This study was a range-finding, nose-only inhalation study designed to determine target concentration levels for a 90-day nose-only inhalation study. Daily 2-hour exposures at 12%, 6%, 3%, and 0% CF_3I were performed for 2 weeks (10 exposures). Suppression of body weight gains, incoordination, and lethargy were significant findings in the high-level animals. Transient body weight suppression was noted in the mid-level animals. Thyroglobulin and reverse T_3 values were increased at all levels. No gross lesions or histopathological (thyroid and parathyroid) effects were noted at necropsy.

INTRODUCTION

Environmental concern over the depletion of stratospheric ozone and global warming has led to an international treaty called the Montreal Protocol (1987) which calls for the phaseout of halons by the year 2000. Presently, the Air Force is using Halon 1301 as a flooding agent for extinguishing in-flight aircraft and electronic equipment fires and for fire extinguishment in confined spaces. Because it is believed to have less ozone-depleting activity, trifluoroiodomethane (CF_3I) is being considered as a possible replacement for Halon 1301.

Little toxicity information is available in the literature concerning CF_3I toxicity. A modified acute inhalation toxicity test was performed in which rats were exposed in a nose-only chamber to 12% CF_3I for 15 min (Skaggs et al., 1993). Salivation was observed in the rats upon removal from the chamber; however, all rats appeared to be fully recovered by 2-hour postexposure. A 15-minute, nose-only inhalation median lethal concentration (LC_{50}) value is 27% CF_3I (Ledbetter, 1994). No deaths occurred following an acute 4-hour nose-only inhalation exposure to either 0.5% or 1.0% CF_3I (Kinkead et al., 1994). Additionally, no treatment-related signs of toxic stress were noted immediately following exposure. Histopathologic examination of selected tissues from animals examined immediately following exposure, 3-day postexposure, or after a 14-day postexposure observation period, showed no lesions of pathologic significance.

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Department of Defense (DoD) instructors may undergo repeated exposure to low level CF_3I vapor during routine training sessions. Although the anticipated use of this compound is in confined areas, such as engine compartments, there is potential for personnel confined to crew compartments to experience acute inhalation exposures from leakage of CF_3I. There is also occupational concern for those filling, storing, and transporting cylinders of CF_3I. The DoD is in the process of developing a complete toxicity profile that includes the results of extensive toxicity testing. Results from subchronic inhalation studies will be useful in providing safety recommendations for personnel assigned to training operations during which CF_3I will routinely be used. The Air Force must assure the safety of this compound in the environment and assure health hazard information is available to the individuals responsible for the safe handling of this compound.

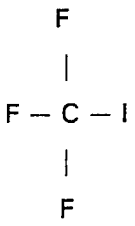
Preliminary to the start of a 90-day nose-only inhalation study, a 2-week range-finding study was performed to identify target organ toxicity, clinical signs of toxic stress, and establish exposure concentrations for the long-term study. This study reports the findings of the 2-week nose-only, range-finding study.

MATERIALS AND METHODS

Test Material

Trifluoroiodomethane is a fluoroalkane. Decomposition products are likely to include hydrogen fluoride and hydrogen iodide. The test material used in this study will be supplied by Pacific Scientific, Tulsa, OK. Pertinent physical and chemical properties follow.

Structure:



CAS No.	2314-97-8
Systematic Name	Iodotrifluoromethane
Molecular Weight	195.91
Empirical Formula	CF_3I
Physical State	Colorless gas
Specific Gravity	2.3608 g/mL (–42 °C)
Melting Point	Not applicable
Boiling Point	–22.5 °C
Flash Point	Not flammable
Vapor Pressure	85 psi @ 20 °C
Solubility in H_2O	Insoluble

CF_3I, a liquid under pressure at room temperature, was obtained from Deepwater, Carson, CA 90746, through Combat Systems Test Activity, Aberdeen Proving Ground, MD. Eleven propane tanks were received each containing between 36 to 69 lbs. The purity analysis supplied by the manufacturer was confirmed to be 99.6+% by gas chromatography/mass spectrometry (GC/MS) (Teckmar 7000 Headspace Analyzer, Forster City, CA; Hewlett-Packard 5890A GC, HP 5970B Mass Selective Detector, and HP Vectra 386/25 Data System, Palo Alto, CA). The supply was also tested for the presence of fluoride ion using a Combination Fluoride Ion Electrode, Model 96-09-00, with an Orion Model 701A Digital Ionalyzer (Orion Research Inc., Cambridge, MA). Using a 1 ppm (w/v) standard as absorber and interpreting the increase in fluoride content similar to a known addition, the material used in the pre-study was found to contain less than 0.5 ppm (w/v) fluoride ion.

Test Animals

Twenty male Fischer-344 (F-344) rats were purchased from Charles River Breeding Laboratories, Raleigh, NC. The rats were 6 weeks of age upon arrival and 9 weeks of age at the initiation of exposures. All rats were identified by tail tattoo and were subjected to a 2-week

quarantine period. Water and feed (Purina Formulab #5002) were available *ad libitum,* except during exposure. Animal room temperatures were maintained at 21 to 25 °C, and the light/dark cycle was set at 12-hour intervals. The animals were single housed in clear plastic cages with wood chip bedding (Betta-Chip, Northeastern Products Corp., Warrensburg, NY). The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the U.S. Department of Health and Human Services (1985).

Contaminant Generation

The CF_3I and the dilution air were delivered from pressurized systems and were controlled through flowmeters. Fine control of the concentration was by minor adjustment of the CF_3I flow in response to analysis. The total air flow provided more than 300 mL/min delivered at each exposure port. Target concentrations of CF_3I during the 2-week study were 12%, 6%, 3%, and 0% (v/v). No oxygen was added to the exposure atmosphere.

A portion of the input air passed through a gas washing bottle (Model 7166-26, Ace Glass, Vinland, NJ) containing water to provide adequate relative humidity to the input air stream. Humidity and temperature of the exposure atmosphere were constantly monitored and recorded using H-CAL dual probes (Models CT830, HY-CAL, Atlanta, GA) and a data acquisition system.

The nose-only chamber used was a stainless steel flow-past chamber as described by Cannon et al., 1983. The chamber has 52 ports; 5 were randomly selected for rat exposure. Plexiglass rat-restraining tubes that extend radially outward were plugged into the ports.

Chamber Analysis

Continuous analysis of the CF_3I was performed using infrared absorption spectrometers (Miran 1A, Foxboro Analytical, South Norwalk, CT). A 10-cm short path cell in combination with a low intensity absorption band at 9.6 to 9.7 microns facilitated the analysis of the high concentrations involved in these studies. Instrumental calibration was performed using known concentrations of CF_3I in air contained in Tedlar sample bags (231 series, SKC, Eighty Four, PA).

TOXICITY ASSESSMENT

Records were maintained of body weights (BW), signs of toxicity, and mortality. Euthanasia was via CO_2 inhalation overdose. At sacrifice, gross pathology was performed on all animals and thyroid and parathyroid tissues were harvested for histopathologic evaluation. Wet tissue weights were determined on heart, liver, kidney, lungs, testes, and thyroid/parathyroid. Tissues were fixed in 10% neutral buffered formalin, trimmed, and further processed via routine methods for hematoxylin-eosin-stained, paraffin-embedded sections. Additionally, blood was drawn for select hematology and

clinical chemistry assays (Table 4.5-1). Erythrocytes were enumerated on a Technician H-1 System (Technician Instruments Corp., Tarrytown, NY), and sera for clinical chemistry evaluations were assayed on an Ektachem 250XR (Eastman Kodak, Rochester, NY). Thyroxine and thyroxine-binding globulin (TBG) assays were performed using a DuPont ACA analyzer (DuPont Co., Wilmington, DE). Additional assays performed were: Radioimmuno Assay for the Quantitative Measurement of Reverse Triiodothyronine in Rat Serum (rT3 RIA^{125} I), and Immunoradiometric Assay for the Quantitative Determination of Rat Thyroglobulin in Serum and Plama (TG^{125} I IRMA CT), both obtained from RADIMTECHLAND, Wien Laboratories, Succasunna, NJ.

TABLE 4.5-1.

Thyroxine (T_4)	White blood cell (WBC)
Triiodothyronine (T_3)	Neutrophil (NEUT)
Thyroglobulin (HTG)	Lymphocyte (LYMP)
Thyroid stimulating hormone (TSH)	Monocyte (MONO)
Reverse T_3 (RT_3)	Eosinophil (EOS)
Free thyroxine index (FTI)	Basophil (BASO)
Alanine aminotransaminase (ALT)	Red blood cell (RBC)
Aspartate aminotransaminase (AST)	Hemoglobin (HGB)
Albumin (ALB)	Hematocrit (HCT)
Globulin (GLOB)	Mean corpuscular volume (MCV)
	Mean corpuscular hemoglobin (MCH)
	Mean corpusclar hemoglobin (MCHC)
	Platelet (PLT)
	Albumin/Globulin ratio ALB/GLOB ratio)

RESULTS

Exposure System Analysis

The specified target concentrations of 12%, 6%, and 3% CF_3I were maintained during the daily 2-hour exposures. The exposure mean concentrations were maintained within ±2% of the desired concentrations. Mean concentrations for each exposure, along with mean high and low daily concentrations, are provided in Table 4.5-2. The relative humidity ranged between 40% and 50%, while the temperature ranged from 68 to 74 °F.

TABLE 4.5-2. CF_3I CONCENTRATIONS INHALED BY MALE F-344 RATS

Target Concentration (%)	12.00	6.00	3.00
Mean Concentration (%)	11.97	6.04	3.00
Standard Error	< 0.01	< 0.01	< 0.01
Lowest Daily Mean (%)	11.86	5.97	2.94
Highest Daily Mean (%)	12.03	6.06	3.03
Mean Temperature (°F)	71.3	71.3	71.3
Mean Relative Humidity (%)	46.4	46.7	46.5

Inhalation Toxicity

There were no deaths resulting from exposure. At the conclusion of each 2-hour exposure, the rats from the high- and mid-level groups appeared lethargic and demonstrated slight incoordination. The rats from the low-level exposure did not show any clinical signs that differed from the control rats. Mean body weight gain of the high-exposure group was statistically significantly ($p < 0.01$) depressed after 7 days and again after 14 days. The mid-exposure group had a significant ($p < 0.05$) depression in mean weight gain after 7 days, but recovered and was not different than controls at 14 days (Table 4.5-3).

TABLE 4.5-3. MEAN BODY WEIGHTS[a] AND MEAN BODY WEIGHT GAINS OF MALE F-344 RATS EXPOSED TO CF_3I VIA NOSE-ONLY INHALATION

	Day of Exposure		
Exposure Group	**0**	**7**	**14**
12%			
Mean BW (g)	186.0	186.3	188.8
Mean BW Gain (g)	—	0.3[b]	2.6[b]
6%			
Mean BW	182.2	184.4	194.2
Mean BW Gain	—	2.3[c]	9.7
3%			
Mean BW	181.9	187.1	198.0
Mean BW Gain	—	5.2	0.9
0 (control)			
Mean BW	182.5	191.1	201.2
Mean BW Gains	—	8.6	10.2

[a] Mean ± SEM, N = 5.
[b] Different from control at $p < 0.01$.
[c] Different from control at $p < 0.05$.

A number of hematology and clinical chemistry values of exposed rats were statistically different than control values (Table 4.5-4). The WBC count was decreased and the albumin values of the high-exposure group were elevated when compared with control values. Neutrophils and platelet counts were high, while lymphocytes, red cell count, and hemoglobin values were low in the mid-exposure rats. Thyroglobulin (HTG) and reverse T_3 (RT_3) values were significantly increased at all exposure levels.

At necropsy, the high-level rats appeared unthrifty grossly. However, no gross lesions were found at any exposure level and no differences were noted in absolute or relative organ weights. Histopathologic examination of thyroids and parathyroid glands determined no differences in CF_3I-exposed rats.

TABLE 4.5-4. HEMATOLOGY AND CLINICAL CHEMISTRY VALUES (MEAN ±SD) MALE RATS FOLLOWING TWO WEEK NOSE-ONLY INHALATION OF CF_3I

	Control	3%	6%	12%
WBC ($X10^3/\mu L$)	9.2 ± 0.4	8.8 ± 0.3	7.8 ± 0.6[b]	7.3 ± 0.2[a]
NEUT	15.9 ± 1.6	19.3 ± 1.4	23.1 ± 1.0[b]	19.5 ± 2.5
LYMP	78.2 ± 1.4	75.2 ± 1.6	71.2 ± 1.6[b]	75.9 ± 1.0
MONO	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.2	1.2 ± 0.1
EOS	0.6 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
BASO	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
RBC ($X10^6/\mu L$)	8.8 ± 0.1	8.7 ± 0.1	8.4 ± 0.1[a]	8.7 ± 0.1
HGB (g/dL)	15.7 ± 0.3	15.3 ± 0.1	14.7 ± 0.2[a]	15.2 ± 0.2
HCT (%)	53.1 ± 1.1	52.1 ± 0.7	50.0 ± 1.1	51.3 ± 0.5
MCV (fl)	59.9 ± 0.5	60.1 ± 0.4	59.7 ± 0.6	58.8 ± 0.4
MCH (pg)	17.7 ± 0.2	17.7 ± 0.1	17.6 ± 0.1	17.4 ± 0.1
MCHC (g/dL)	29.6 ± 0.2	29.4 ± 0.2	29.4 ± 0.4	29.6 ± 0.1
PLT	630.2 ± 11.1	642.2 ± 22.1	724.2 ± 20.7[a]	690.6 ± 38.1
TP (g/dL)	6.5 ± 0.1	6.5 ± 0.1	6.5 ± 0.1	6.7 ± 0.1
ALB (g/dL)	3.7 ± 0.1	3.7 ± 0.1	3.4 ± 0.1	4.0 ± 0.1[a]
GLOB (g/dL)	2.8 ± 0.0	2.8 ± 0.0	2.8 ± 0.1	2.8 ± 0.0
AST (IU/L)	94.4 ± 3.0	93.6 ± 3.4	88.6 ± 1.9	85.6 ± 5.0
ALT (IU/L)	46.6 ± 5.2	45.6 ± 3.2	43.8 ± 3.2	39.4 ± 2.4
TSH	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
T4	3.4 ± 0.1	5.2 ± 0.1[b]	4.0 ± 0.4	3.8 ± 0.2
T3	40.6 ± 0.4	41.7 ± 0.5	42.0 ± 0.4	41.5 ± 0.2
FTI	1.4 ± 0.0	2.2 ± 0.1[c]	1.7 ± 0.2	1.6 ± 0.1
ALB/GLOB ratio	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	1.4 ± 0.0[b]
HTG (NGM/ML)	0.2 ± 0.0	0.3 ± 0.0[b]	0.5 ± 0.0[c]	0.5 ± 0.0[c]
RT3 (NGM/dL)	5.5 ± 0.3	6.9 ± 0.2[b]	7.0 ± 0.2[b]	7.7 ± 0.4[c]

[a] Significantly different than Control at $p < 0.05$.
[b] Significantly different than Control at $p < 0.01$.
[c] Significantly different than Control at $p < 0.001$.
N = 5.

DISCUSSION

This was a range-finding study, the results of which were to be used to determine concentration levels for use in a 90-day study. The failure of the high-level (12%) group to gain weight over the 2-week period, as well as the significant clinical signs of stress and increases in HTG and RT3, indicated that a 12% concentration would be inappropriate for a 90-day study. The mid-level (6%) group showed some clinical signs of stress, transient weight gain suppression, and increases in HTG and RT3. The HTG and RT3 values were increased in the low-level (3%) animals, but the increase is not believed to be biologically significant.

Recommended CF_3I concentrations for the 90-day study are 8%, 4%, and 2%. The high level should produce clinical signs and demonstrate effects on the thyroid glands but no fatalities. The mid level should result in minimal effects and the low level is expected to be a no observable effect level (NOEL).

REFERENCES

Cannon, W. C., E.F. Blanton, and K.E. McDonald. 1983. The Flow-Past Chamber: An Improved Nose-Only Exposure System for Rodents. *Am. Ind. Hyg. Assoc. J.* 44(12):923 –933.

Kinkead, E.R., S.A. Salins, R.E. Wolfe, H.F. Leahy, and J.H. English. In Press. *Acute Toxicity Evaluation of Halon Replacement Trifluoroiodomethane (CF_3I).* AL-TR-1994, Armstrong Laboratory, Wright-Patterson Air Force Base, OH.

Ledbetter, A. 1994. Personal communication.

Skaggs, S.R., D.S. Dierdorf, and R.E. Tapscott. 1993. *Update on Iodides as Fire Extinguishing Agents.* International CFE and Halon Alternatives Conference, Washington, DC. pp. 800–808.

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5.1 PHARMACOKINETICS OF HCFC-123 IN DOGS

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INTRODUCTION

HCFC-123 is used primarily as a foam blowing agent, as a refrigerant, and in cleaning solvents. The Air Force is considering the use of HCFC-123 as a fire extinguishant, replacing Halon 1211. Halon 1211 has been used as a fire extinguishant in streaming systems, where the extinguishant is manually discharged through a nozzle of small, portable units that are commonly found in industry, military, and office settings. Jarabek et al. (1994) reviewed the process of searching for CFC substitutes with HCFC-123 as a specific example.

Potential occupational military exposures with HCFC-123 as a fire extinguishant include maintenance personnel (crew chiefs) responding to aircraft fires on the flight line or in a large indoor structure, such as an aircraft hangar, and trained fire fighters responding to alarms. The fire fighter exposure scenario deals with military personnel who don appropriate fire fighting gear, including respirators, immediately prior to fighting fire. Thus, the exposure scenario of concern for the Emergency Exposure Guidance Level involves the emergency situation where maintenance personnel attempt to put out a fire without availability of the appropriate fire fighting equipment. The exposure duration of concern involves a 1-minute period to simulate personnel discharging either the entire contents of a small (1- or 3-lb) extinguisher or the partial contents of a large (150-lb) extinguisher while attempting to put out an aircraft fire (usually an engine fire) from upwind of the fire (Dr. Kibert, WL/FIVS, personal communication).

The U.S. EPA has been mandated, under Title VI of the U.S. Clean Air Act of 1990 (Public Law 101-549), to evaluate alternatives to Class I ozone-depleting substances, including halon fire and explosion protection agents. Under the Significant New Alternatives Policy Program, the EPA considers the cardiac sensitization potential as the most sensitive biological endpoint because of the potent sensitizing effect of this chemical and similar chemicals in the epinephrine-challenged dog model. For HCFC-123, the EC_{50} (95% confidence interval) was determined by Trochimowicz and Mullin (1973) to be 1.9% (1.29% to 2.82%) for a 5-minute exposure. Dogs exposed to 10,000 ppm for 5 min show no "marked" arrhythmias, and this can be considered to be the no observable adverse effect level (NOAEL). Converting this 5-minute NOAEL to a 1-minute NOAEL is not straightforward. Haber's Law (Haber, 1924) suggests that the 1-minute NOAEL would be 5%, because the product of concentration and time is a constant. Haber's law is applicable to a limited

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number of situations in which all processes are linear and the toxic moiety is the parent chemical. Results of the dog pharmacokinetic study described above, show that Haber's law is not followed in this situation. Cardiac sensitization might be explained by a chemical interfering with neural transmission in the heart. The mechanism of action would be expected to be similar to the physical effects of anesthetics which act at the membrane level by changing fluidity according to their affinity to interact with lipids. This phenomenon is proportional to concentration. From these assumptions, it follows that it is important to know the concentration in the heart or blood at the time of the arrhythmias.

The objective of this study was to evaluate the pharmacokinetic behavior of HCFC-123 in an exposure scenario that mimics the cardiac sensitization test in dogs and use the data to support the development of a PBPK model. Specific emphasis was placed on the measurement of blood and tissue samples following exposure of 1% or 5% HCFC-123 for 1 to 5 minutes duration.

METHODS

Test Material

1,1-DICHLORO-2,2,2-TRIFLUOROETHANE (HCFC-123)

Physical and Chemical Properties

Chemical Formula:	$CHCl_2CF_3$
Molecular Weight:	152.9
Synonyms:	HCFC-123
CAS No.:	306-83-2
Physical state:	Liquid at normal temperatures
Boiling point:	27.9 oC @ 760 mm Hg
Freezing point:	−107 °C
Vapor pressure:	11 psi (20 °C)
Vapor density:	(Air = 1) 3.6
Solubility in water:	0.21% (wt) @ 70 °F
Flash point:	N.A. — No flash point
Auto ignition:	Unknown, probably not applicable
Flame limits:	(In air, % by vol), none

General Procedure

Two male beagle dogs per time-point were exposed to HCFC-123 at either 1% or 5% for various exposure durations (Table 5.1-1). Exposure was "nose-only" via a specially adapted canine anesthesia mask equipped with a two-way non-rebreathing valve. The exposure system was designed to provide instantaneous exposure of the dogs to the target concentrations and permit the drawing of blood samples. Blood samples were collected (as applicable) at 0 (preexposure), 1, 2, 3, 4, 5, 7.5, 10, 15, 30, 45, and 60 min (during exposure), and 1, 3, 6, 16, and 31 min (postexposure recovery) and analyzed for HCFC-123. At the end of the exposure periods (or postexposure recovery periods), animals were euthanized and samples from selected tissues (heart, liver, fat, and skeletal muscle) were collected as rapidly as possible for analysis of HCFC-123.

TABLE 5.1-1. EXPERIMENTAL DESIGN

Number of Dogs	Dog I.D. Number	Exposure Concentration (HCFC-123)	Exposure Time	Postexposure Time
2	1974 / 1999	1%	1 min	na
2	1975 / 1986	1%	5 min	na
2	1992 / 1995	1%	60 min	na
2	1993 / 1994	1%	60 min	30 min
2	1979 / 1990	5%	1 min	na
2	1983 / 1997	5%	5 min	na

na = not applicable.

Exposure System

Liquid HCFC-123 was evaporated by heating a glass reservoir, while air passed across the test article surface. The HCFC-123 was first brought to target concentrations in a 500 L NYU-type inhalation chamber, then supplied to the animal via a sideport. Concentrations in the exposure chamber were monitored with a Miran 1A Gas Analyzer. Chamber air flow, temperature, relative humidity, and oxygen were monitored as well. Each animal was exposed individually. The animal was first secured in a sling, and the snout placed in a modified dog anesthesia mask. The snout went through a small hole in a rubber diaphragm to provide a seal. The animal breathed either chamber atmosphere or room air via a valve on the exposure line sideport. The animal breathed the HCFC-123 through a two-way non-rebreathing valve to maintain a unidirectional flow of chemical.

Blood and Tissue Sampling

A 5.0 cm over-the-needle teflon catheter was inserted into a saphenous vein. The catheter was attached to a three-way valve so that heparinized saline could be used to flush the catheter. A three-mL glass syringe was used to draw blood samples. Three, approximately 100 mL samples were place into preweighed headspace vials and reweighed for analysis of HCFC-123 concentration. For tissue sampling, animals were euthanized by lethal injection. The dead animals were transferred as rapidly as possible to a necropsy suite to harvest tissues for HCFC-123 analysis. The intact heart was removed first, followed by samples of fat (perirenal), liver, and skeletal muscle. For each tissue, three subsamples of approximately 500 mg were weighed and sealed in headspace vials. In general, the entire necropsy procedure was completed in less than 5 minutes.

Analysis of Blood and Tissue Samples for HCFC-123

Blood and tissue samples were stored in a −80 °C freezer until analysis. Headspace vials containing blood or standards were loaded onto a Tekmar 7050 static headspace sampler for injection onto a Varian 3700 Gas Chromatograph. The gas chromatograph was equipped with a 0.53 mm × 25 m PoraPlot Q column and an electron capture detector. Tissue samples were first digested with sodium hydroxide solution to release the HCFC-123 into the headspace. The digestion process occurred within the headspace vial. The digested samples were analyzed in the same manner as the blood. Sample headspace HCFC-123 concentrations were calculated from a standard curve.

RESULTS

The blood and tissue (heart, muscle, liver and fat) concentrations for all exposure scenarios are given in Tables 5.1-2 and 5.1-3, respectively. Results for exposure durations of 1 to 5 min are shown in a summarized form in Table 5.1-4. In animals exposed for 60 min (n = 4), the maximum venous blood concentrations (mean values) were attained within 30 min, with less than a 3% increase over the next 30 min (Figure 5.1-1 and Table 5.1-2). Animals allowed to recover for 30 min (n = 2), had rapid decreases in the venous blood concentrations within the first 16 min with concentrations approaching the limits of detection by 31 min postexposure (Figure 5.1-2 and Table 5.1-2).

Figures 5.1-3 and 5.1-4 are graphs of the triplicate blood concentrations at the early time points for all animals exposed to 1% and 5% HCFC-123, respectively. The experimental design allowed for the sampling of 8 animals at 1.0 minute during the 1% exposure. Due to problems in sampling, half of the 1.0-minute samples were not available for analysis.

The rise and fall in tissue concentrations paralleled that of blood. Heart, liver and muscle tissue appeared to take up HCFC-123 much quicker than fat tissue (Table 3). It should be noted that the concentration of chemical in fat, in animals exposed for 60 minutes, was 3 to 5 fold higher than the other tissues as expected. The solubility of HCFC-123 in fat (partition coefficient = 52.9) is approximately 25 times greater than muscle (2.3), liver (1.9) or heart (2.5) tissues. Subsequently, the washout of chemical in fat tissue was much slower than any other tissue. In general, the blood and tissue concentrations of HCFC-123 both increased with exposure time and increasing concentration.

TABLE 5.1-2. BLOOD CONCENTRATIONS IN DOGS EXPOSED BY INHALATION TO HCFC-123

1% Exposure Concentration	Time of Exposure (Min) 1	2	3	4	5	7.5	10	15	30	45	60	Animal I.D.
	7.6	10.0	11.0	11.7	9.5	7.6	7.2	10.3	22.7	13.8	15.7	1993
	—	3.3	8.8	6.6	9.1	8.2	13.6	26.1	31.3	36.1	34.5	1994
	4.4	4.4	3.8	4.9	5.4	10.2	12.0	24.7	33.2	33.5	33.6	1992
Blood	4.1	10.6	16.5	18.2	18.4	17.2	14.3	21.1	22.9	27.3	29.1	1995
Concentrations	—	0.7	1.5	2.3	2.7	—	—	—	—	—	—	1975
(mg/L)	—	3.3	6.0	7.3	—	—	—	—	—	—	—	1986
	4.7	—	—	—	—	—	—	—	—	—	—	1999
	—	3.8	—	—	—	—	—	—	—	—	—	1974
Mean	5.2	5.2	4.6	8.5	9.0	10.8	11.8	20.6	27.5	27.8	28.2	

5% Exposure Concentration	Time of Exposure (Min) 1	2	3	4	5	Animal I.D.
Blood	5.8	13.0	21.5	40.5	84.4	1997
Concentrations	7.8	109.9	131.2	125.2	145.4	1983
(mg/L)	43.2	—	—	—	—	1979
	28.9	—	—	—	—	1990
Mean	21.4	61.4	76.3	82.8	114.9	

1% Exposure Concentration	Time Post 60 Minute 1% Exposure (Min) 1	3	6	16	31	Animal I.D.
Blood (mg/L)	14.0	9.4	7.6	5.3	4.0	1993
Concentrations	30.6	27.8	25.1	6.7	—	1994
Mean	22.3	18.6	16.3	6.0	4.0	

TABLE 5.1-3. TISSUE CONCENTRATIONS IN DOGS EXPOSED BY INHALATION TO HCFC-123

Exposure Concentration	Tissue Concentrations (mg/L)				Animal
Exposure Time	Heart	Muscle	Liver	Fat	I.D.
One Percent	14.7	13.8	12.9	2.1	1999
One Minute	6.7	7.0	5.1	0.6	1974
One Percent	15.8	5.5	14.6	15.9	1975
Five Minutes	18.6	7.2	19.5	13.9	1986
One Percent	39.4	34.7	51.1	199.1	1992
Sixty Minutes	37.9	66.6	46.0	182.2	1995
One Percent	2.5	5.3	2.2	118.5	1993
Sixty Minutes[1]	2.6	10.2	3.6	195.3	1994
Five Percent	107.4	24.9	75.8	3.9	1979
One Minute	94.8	29.1	48.2	9.7	1990
Five Percent	141.0	39.5	81.8	78.3	1997
Five Minutes	179.8	36.4	174.9	46.2	1983

Samples collected thirty minutes postexposure.

TABLE 5.1-4. BLOOD AND TISSUE CONCENTRATIONS[1] IN DOGS EXPOSSED BY INHALATION TO 1% OR 5% HCFC-123. The number of dogs exposed was two unless noted otherwise.

	One Percent		Five Percent	
Sample	**1 minute**	**5 minutes**	**1 minute**	**5 minutes**
Blood	5.2 (4.1-7.6)[2]	9.0 (2.7-18.4)[3]	21.4 (5.8-43.2)[2]	114.9 (84.4-145.4)
Heart	10.7 (6.7-14.7)	17.2 (15.8-18.6)	101.1 (94.9-107.4)	160.4 (141.0-179.8)
Muscle	10.4 (7.0-13.8)	6.4 (5.5-7.2)	27.0 (24.9-29.1)	38.0 (36.4-39.5)
Liver	9.0 (5.1-12.9)	16.8 (14.4-19.2)	62.0 (48.2-75.8)	128.3 (81.8-174.9)
Fat	1.36 (0.6-2.1)	14.9 (13.9-15.9)	6.8 (3.9-9.7)	62.3 (46.2-78.3)

[1] concentrations in mg/L expressed as mean (range).
[2] number of dogs was 4.
[3] number of dogs was 5.

Cardiac sensitization studies of the dog, conducted by Trochimowicz and Mullin (1973), challenged the dog with epinephrine after a 5-minute exposure to HCFC-123. In an attempt to better extrapolate this 5-minute exposure to a 1-minute exposure, of concern for an emergency exposure guidance level (EEGL), the inhalation exposure was conducted as described, with a small number of dogs, designed to mimic the 5-minute exposure for the standard cardiac sensitization test (no epinephrine given) and provide blood and tissue samples after a 1-minute exposure.

Analysis of the pharmacokinetics from this study facilitate the extrapolation of the 5-minute cardiac sensitization level to a 1-minute cardiac sensitization level. As expected, blood and tissue concentrations both increased with exposure time and increasing concentration. Blood and tissue concentrations after a 5-minute exposure to 1% HCFC-123 (the NOAEL) can be assumed to be the HCFC-123 concentrations at which there would be no cardiac arrhythmias. Blood concentrations and heart tissue concentrations are considered to be most relevant to the end point of cardiac sensitization. Table 5.1-4 shows that Haber's Law is not appropriate for this situation, because according to Haber's Law, 1% HCFC-123 for 5 min should be equivalent to 5% HCFC-123 for 1 min. The blood concentration at 5% HCFC-123 for 1 min was approximately 2.5 times greater than 1% for 5 min and the heart tissue concentration was approximately 6 times greater in the higher concentration, shorter duration exposure than in the lower concentration, longer duration exposure.

Potential occupational military exposure to HCFC-123 as a fire extinguishant is expected to be brief with low probability of repeated exposures. The selection of a 1-minute exposure duration for establishing an EEGL concentration is appropriate and represents a "typical" emergency exposure scenario outdoors or indoors where the area can be evacuated after use of a fire extinguisher. For HCFC-123, the endpoints of pharmacologic or adverse effects considered for establishing an EEGL are cardiac sensitization, anesthesia or CNS-related effects, malignant hyperthermia, and hepatotoxicity. The problem with all of these endpoints is extrapolating from studies having long exposure times, in which no effect levels were determined, to a short 1-minute exposure. An HCFC-123 concentration of 19,000 ppm (1.9%) is recommended as a 1-minute EEGL based on cardiac sensitization, the most sensitive endpoint.

Blood concentrations measured in dogs after exposure to 1% HCFC-123 for 5 min (the NOAEL) averaged 9.0 (range 2.7-18.4) mg/L. If we assume that sensitization will not occur if this concentration is not exceeded (Beck et al., 1973), we can use the slope of the 1-minute blood concentrations at 1% and 5% to estimate the exposure concentration required to reach this blood concentration after a 1-minute exposure. This value turns out to be 1.9% or 19,000 ppm (Figure 5.1-5).

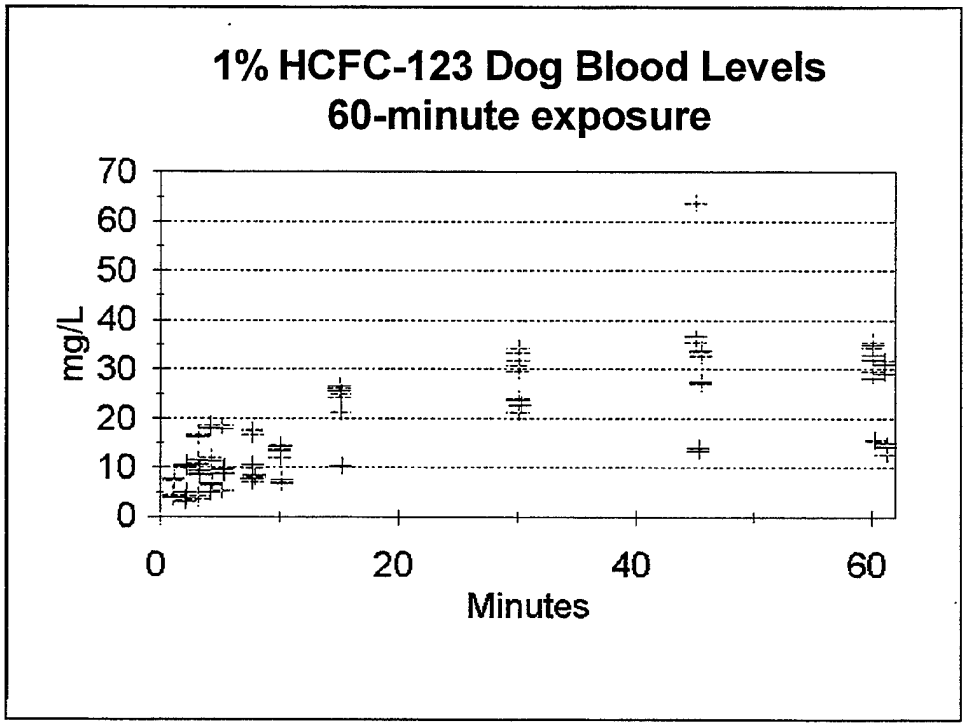

Figure 5.1-1.

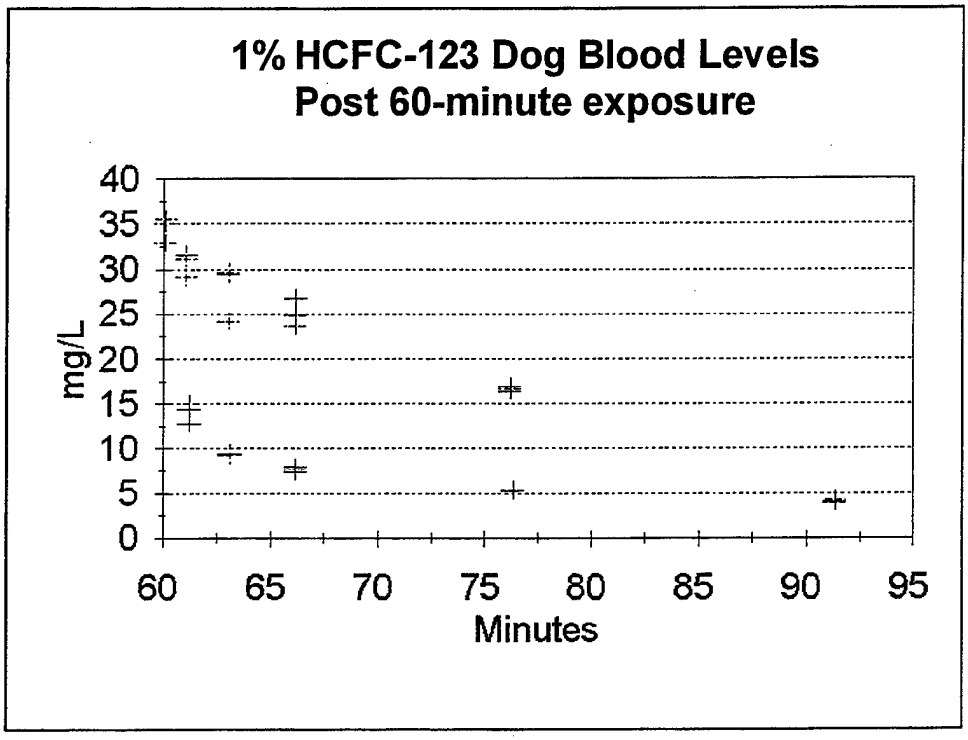

Figure 5.1-2.

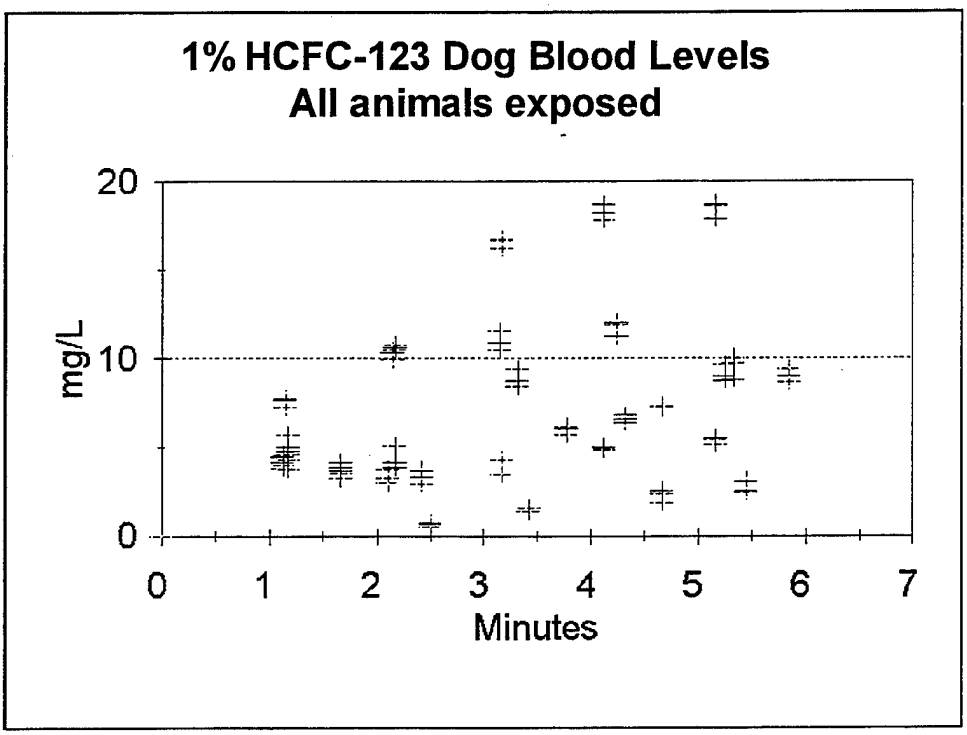

Figure 5.1-3.

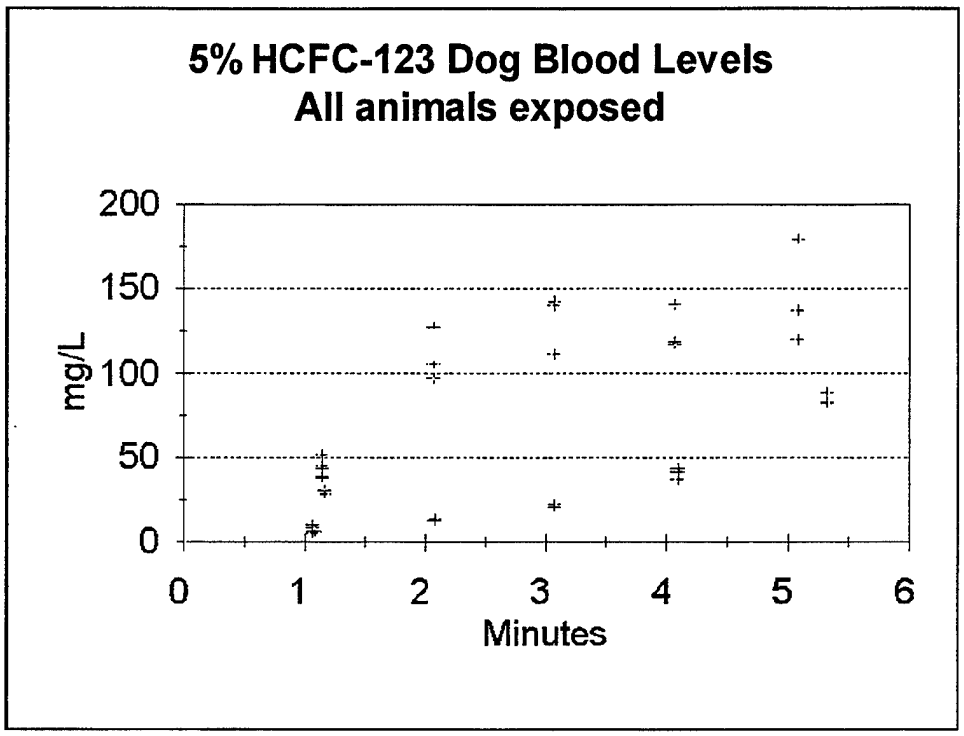

Figure 5.1-4.

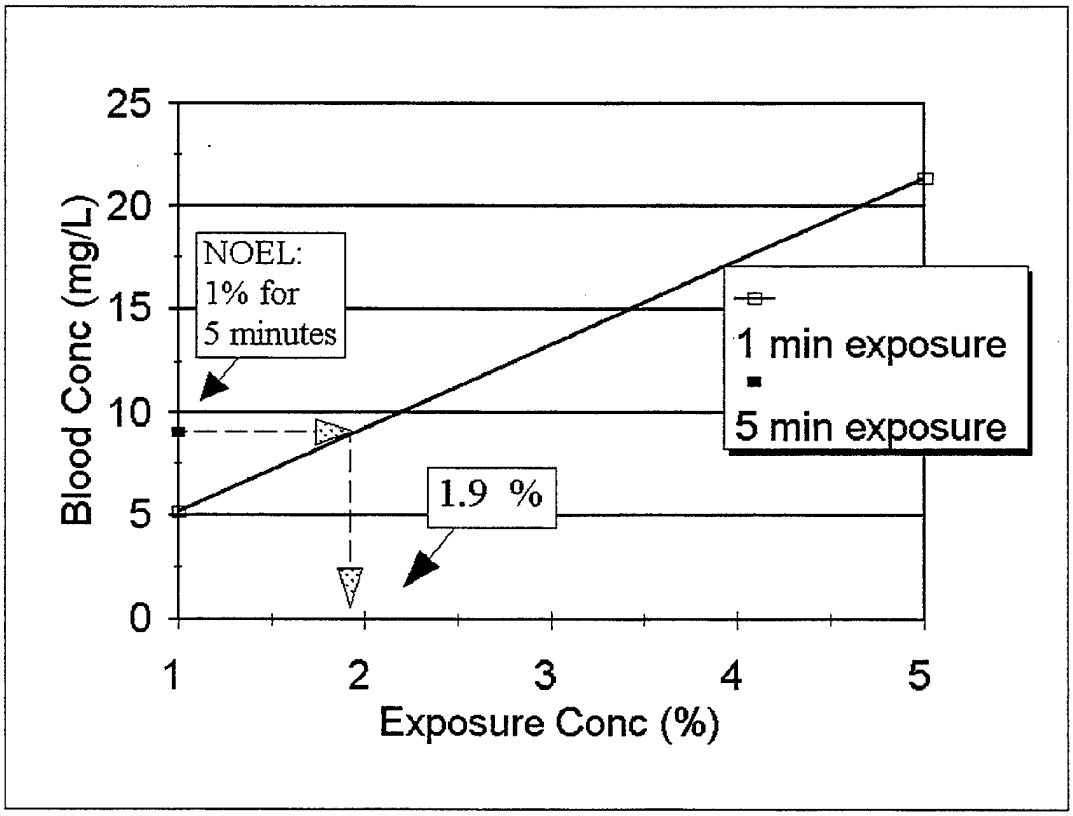

Figure 5.1-5. Extrapolation from the measured blood concentration at the NOEL. The 1-minute line connects blood concentrations measured after a 1-minute exposure to 1% and 5% HCFC-123.

The EEGL based on cardiac sensitization makes several conservative assumptions. First, the dog cardiac sensitization model incorporates priming and challenge doses of epinephrine which are supraphysiological, or about 10 times that of human adrenal release during times of stress. The original purpose of this test was to rank chemicals which might be cardiac sensitizers. In this test, the epinephrine dose is maximized to be just below the point where it causes arrhythmias by itself. DuPont (Trochomowicz, personal communication) has compared the cardiac sensitization test with epinephrine to tests where exogenous epinephrine was replaced with a "treadmill" or "fright". In these tests, the test chemical concentration required to produce the same effect was 2 to 4 times that required in the epinephrine-challenge test. Second, the EEGL is set based on the NOEL, which is a function of the doses chosen for the test. The EEGL would be higher if it were based on the lower 95% confidence interval of the EC_{50}, or blood concentrations at 1.29%.

Although the mechanism of action of cardiac sensitization is unknown, there is good evidence that like, anesthetic effects, the concentration of chemical in the membrane causes the effect (Clark and Tinston, 1982). The physical rather than chemical mechanism of action argues against using safety factors for the EEGL based on cardiac sensitization (Bellin, 1992).

Laboratory animal studies indicate that HCFC-123 is approximately three times less potent than halothane for producing anesthesia. Concentrations of approximately 1% halothane produce anesthesia. A subanesthetic exposure of 4,000 ppm halothane for 30 min causes transient impairment in mental performance in humans. In HCFC-123 inhalation studies, mild CNS depression is observed in rats exposed (for 6 h) to 5,000 ppm, but these effects are rapidly reversible upon cessation of exposure. Dogs exposed to 10,000 ppm HCFC-123 for 5 min show signs of CNS depression (details were not given). In studies performed by Clark and Tinston (1982) involving acute exposure of rats and dogs to a series of halogenated hydrocarbons, CNS effects and cardiac sensitization appear together at the same concentration, and the more potent a chemical to produce CNS effects, the more potent it was to produce cardiac sensitization. For select halogenated hydrocarbons, it would be difficult to choose one of these biological endpoints over the other as the most sensitive measure of pharmacological or toxicological effect. For the purposes of the EEGL it was assumed that anesthetic effects would occur at the concentration which might cause cardiac sensitization.

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REFERENCES

Beck, P.S., D.G. Clark, and D.J. Tinston. 1973. The pharmacologic actions of bromochlorodifluoromethane (BCF). *Toxicol. Appl. Pharmacol.* **24**:20–29.

Bellin, J.S. 1992. Supporting document for evaluation of the significance of cardiac sensitization. prepared for USEPA Global Change Division under Contract 68D90068: WA 3–20 by ICF Incorporated, Fairfax VA.

Clark, D. G. and D.J. Tinston. 1982. Acute inhalation toxicity of some halogenated and non-halogenated hydrocarbons. *Human Toxicol.* **1**:239–247.

Haber, F. 1924. Zur Geschichte des Gaskrieges. In *Fünf Vortraege aus den Jahren 1920–1923*, pp. 75–92. Verlag Julius Springer, Berlin.

Jarabek, A.M., J.W. Fisher, R. Rubenstein, J.C. Lipscomb, R.J. Williams, A. Vinegar, and J.N. McDougal. 1994. Mechanistic insights aid the search for CFC substitutes: Risk assessment of HCFC–123 as an example. *Risk Analysis* **14**:231–250.

Trochimowicz, H.J. and L.S. Mullin. 1973. Cardiac sensitization potential (EC_{50}) of trifluorodichloroethane. Haskell Laboratory Report No. 132–73.

5.2 METABOLISM OF 2,2-DICHLORO-1,1,1-TRIFLUOROETHANE (HCFC-123) BY HUMAN HEPATIC MICROSOMES

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INTRODUCTION

Increasing atmospheric levels of chlorofluorocarbons (CFCs) have resulted in stratospheric ozone depletion (Molina and Rowland, 1974). As a result, the Montreal Protocol of 1987 called for a phaseout of CFCs by the year 2000. One of these CFCs, Halon 1211, is commonly used as a fire extinguishant. One candidate considered as a replacement for Halon 1211 is 2,2-dichloro-1,1,1-trifluoroethane (HCFC-123), an analog of the anesthetic halothane. The metabolism of halothane has been described both *in vivo* and *in vitro* (Van Dyke and Gandolfi, 1976; Cousins et al., 1979), but the metabolism of HCFC-123 has not been as extensively studied, and no metabolism studies in humans have been reported. A single *in vivo* metabolism study, in which rats were exposed to 1% HCFC-123 for 2 h, has shown that HCFC-123 is oxidatively metabolized to trifluoroacetic acid (TFA). In that study levels of trifluoroacetylated liver proteins were nearly identical to those detected after an exposure to 1% halothane for 2 h (Harris et al., 1991). These liver protein adducts are formed from a trifluoroacetyl halide intermediate arising from the oxidative metabolism of the substrate and are believed to be involved in the development of halothane-induced hepatitis in humans (Pohl et al., 1989; Satoh et al., 1989). Because the structure of HCFC-123 is similar to that of halothane, and because the potential for environmental and occupational exposure to HCFC-123 exists, the rates of HCFC-123 metabolism by human hepatic microsomes were assessed as part of the safety assessment of this chemical.

MATERIALS AND METHODS

HCFC-123 (CAS No. 306-83-6) was supplied by Allied Signal, Inc. (Morristown, NJ) and was found to contain about 5% 1,2-dichloro-1,1,2-trifluoroethane as an impurity. All other reagents were obtained from Sigma Chemical Co. except as specified. The human liver specimens used in this study were obtained from Stanford Research Institute, Menlo Park, CA. Liver specimens from organ donors were stored at −135 °C, shipped frozen, and stored at −70 °C until used for the preparation of microsomes. Prior to the original acquisition of the livers, the organs had been perfused in preparation for possible organ transplant. The livers were negative for the presence of hepatitis A and B viruses as well as human immunodeficiency virus.

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Livers were thawed at room temperature and homogenized in 4 vol of ice-cold 0.154 M KCl/0.05 M Tris-HCl (pH 7.4). The homogenate was centrifuged at 4 °C at 500 and 10,000 × g for 10 min each. The supernatant fraction, obtained after the final spin, was centrifuged at 104,000 × g for 60 min at 4 °C, to obtain the microsomal pellet. The pellet was washed with and resuspended in the 0.154 M KCl/0.05 M Tris-HCl(pH 7.4) prior to storage at -80 °C. Protein concentrations were determined by the BCA method (Pierce Chemical Co., Rockford, IL). The cytochrome P450 content of sodium dithionite-reduced microsomes in 0.1M Tris-HCl (pH 7.6) was determined by the method of Omura and Sato (1964).

Initial experiments were conducted to establish conditions leading to linear reaction rates of TFA formation with respect to time and protein concentration. Experiments also were conducted to determine the optimal pH and saturating substrate concentration. Microsomal incubations were conducted in 25 mL Erlenmeyer flasks sealed with Teflon-lined silicon septa. The headspace atmosphere was prepared by mixing compressed air and nitrogen with gas-flow controllers (Dwyer Instruments, Michigan City, IN) to yield an oxygen concentration of 5%. The headspace of each flask was purged with this gas mixture, which was chosen because the hepatic vein of humans contains approximately 4% to 5% oxygen (Nauck et al., 1981). Microsomes were thawed, diluted with 0.1 M Tris buffer (pH 7.0) to give the appropriate protein concentration, and bubbled for 2 min with the same gas mixture used to purge the flasks. A 2-mL volume of the diluted microsomal suspension was added to each flask with a gas-tight syringe, HCFC-123 was added to the headspace of each flask with a Hamilton syringe, and the flasks were preincubated at 4 °C with vigorous shaking for 15 min. The reactions were initiated by the injection of 25 μL of ß-NADPH solution through the septum to yield a final concentration of 1 mM, and terminated after the appropriate incubation period by rapidly heating the flasks to 60 °C.

For physiological metabolism studies, incubations were conducted as described above. Incubations contained 2 mg/mL of human hepatic microsomal protein in 0.1 M Tris buffer adjusted to a pH of 7.37. HCFC-123 (7.3 μL) was added to the flasks that were incubated for 7 min following the addition of ß-NADPH. This amount of HCFC-123 resulted in a 1.13 mM concentration in solution as quantitated from determining the partitioning of HCFC-123 into the reaction mixture. This concentration (1.13 mM) was derived from physiologically based pharmacokinetic (PBPK) estimates of the steady-state concentration of HCFC-123 in human liver following an exposure to 1% (v/v) of HCFC-123.

The supernatants of all incubations were analyzed for TFA by derivitization to form volatile methyl esters using the method of Maiorino et al. (1980). The chromatographic separation of the TFA-methyl ester was conducted according to the method described by Brashear et al. (1992).

RESULTS

As shown in Figure 5.2-1A, the optimal pH value was approximately 7.0 for the oxidative metabolism of HCFC-123 to TFA. Therefore, 0.1 M Tris buffer at this pH was used for all subsequent optimization experiments. The rate of TFA formation with respect to time is presented in Figure 5.2-1B. The rate was approximately linear for the first 7 min, and became distinctly nonlinear between 7 and 60 min. A time of 7 min was chosen for determination of optimal rates of TFA formation. The effect of increasing the amount of microsomal protein is presented in Figure 5.2-1C. The reaction is apparently nonlinear over the entire range of concentrations, but a concentration of 2 mg/mL was chosen for determination of optimal rates of TFA formation.

When HCFC-123 was introduced into the headspace of the flasks, the amount of TFA formed (normalized per mg of microsomal protein) increased in response to increasing halocarbon concentration (Figure 5.2-2) up to a concentration of 36% (v/v). However, the relationship was nonlinear indicating possible substrate saturation. Above a concentration of 36% in the headspace, the rate of TFA formation was apparently suppressed. Although there is no clear evidence of substrate saturation, a double reciprocal transformation of the first 4 data points on this curve suggested an apparent maximum velocity of 4 nmoles TFA/mg protein/20 min and a half-maximal substrate concentration of 2.9% (v/v) HCFC-123 (Figure 5.2-2 inset).

The linear rates of TFA formation from HCFC-123 conducted under optimal and physiological conditions are presented in Table 5.2-1. Under conditions of physiological pH, and with substrate concentrations in the liver representing those expected under steady-state conditions, the metabolism of HCFC-123 to TFA was approximately 67% of that observed under optimal conditions.

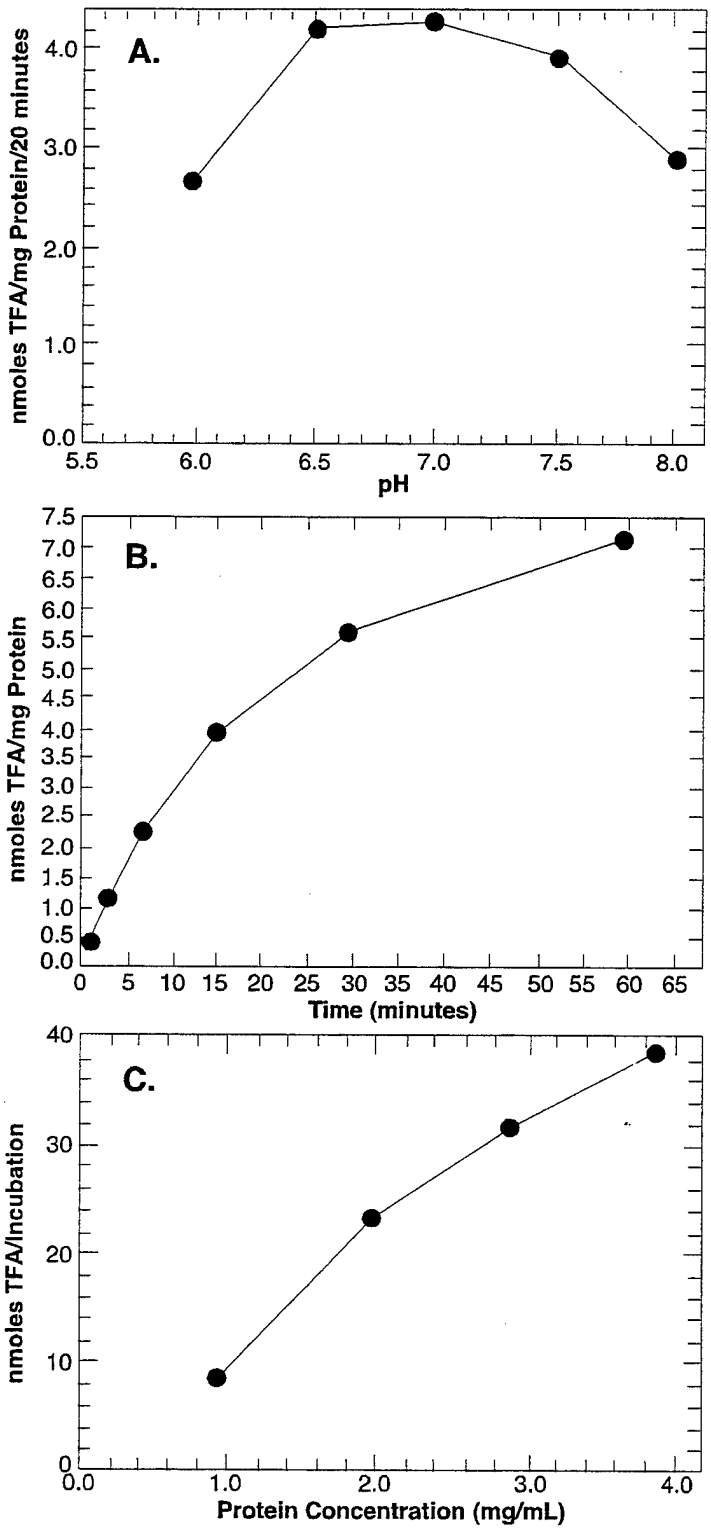

Figure 5.2-1. (A) Effect of pH on the Rate of TFA Formation from HCFC-123 in Human Hepatic Microsomes. Data are from a single experiment. (B) Time Course of the Formation of TFA from HCFC-123 in Human Hepatic Microsomes. Data are from a single experiment. (C) Effect of Human Hepatic Mcrosomal Protein Concentration on the Formation of TFA from HCFC-123. Data are from a single experiment

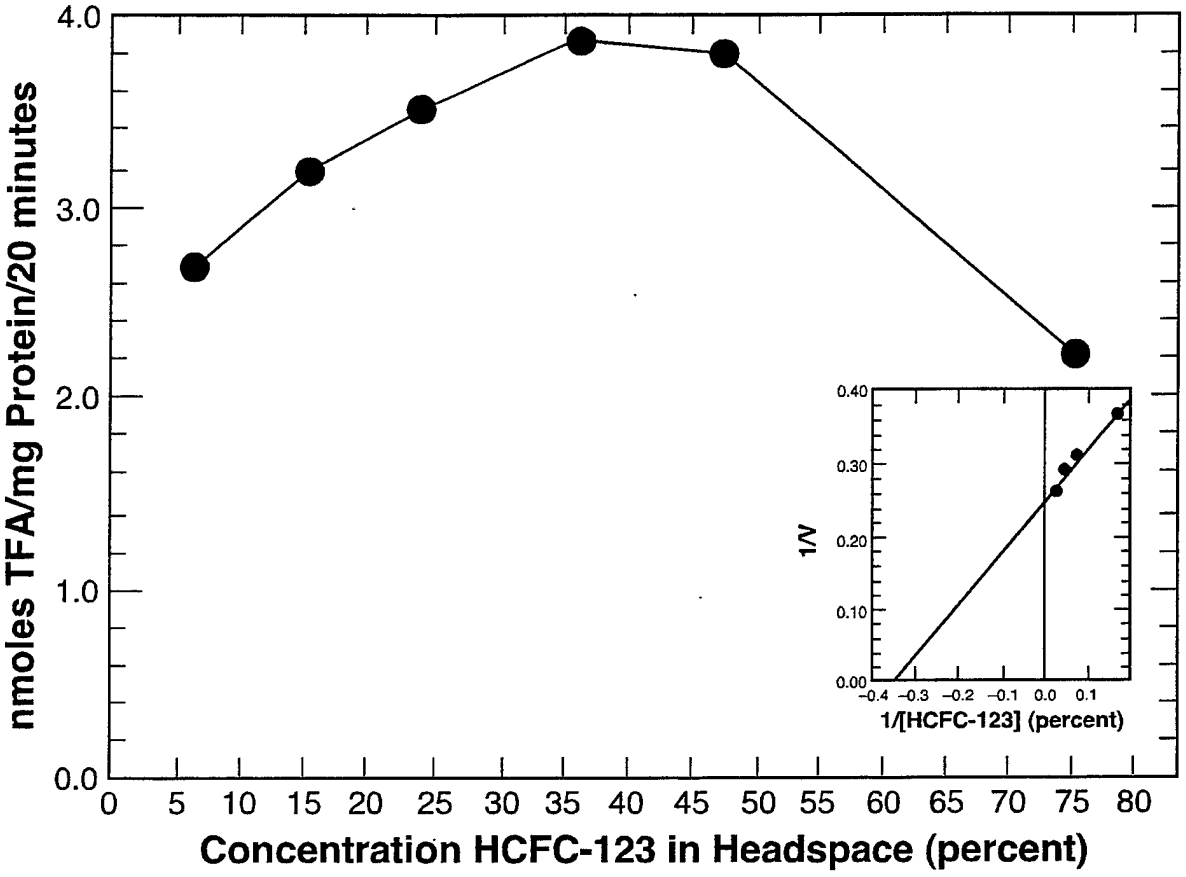

Figure 5.2-2. Substrate-Dependent Kinetics of TFA Formation from HCFC-123 by Human Liver Microsomes. **Data are from a single experiment. Inset; double reciprocal plot of the first 4 points. (Apparent maximum rates and half-maximal substrate concentrations are provided in the *Results* section).**

TABLE 5.2-1. COMPARISON OF PHYSIOLOGICAL AND OPTIMAL RATES OF TFA FORMATION.

Subject Cytochrome P450	(nmole/ mg protein)	Rate of product formation (nmoles/mg protein/min)[a]	
		Optimal	Physiological
H-27	1.01	0.41 (0.39, 0.43)	0.27 (0.29, 0.24)
H-62	0.59	0.33 (0.34, 0.32)	0.24 (0.20, 0.28)
H-64	0.44	0.24 (0.20, 0.27)	0.14 (0.15, 0.13)
Mean ± SD	0.68 ± 0.30	0.33 ± 0.09	0.22 ± 0.07

[a]Values represent the average of duplicate incubations conducted on separate days. The individual values for each subject are given in parentheses.

DISCUSSION

The biotransformation of halothane has been reported in several animal species *in vitro* (Gruenke et al., 1988; Ghantous et al., 1990; Nakao et al., 1991). Although studies describing the metabolism of halothane by humans *in vivo* have been reported (Rehder et al., 1967; Cohen et al., 1975), there are only limited reports concerning the metabolism of halothane by humans *in vitro* (Gruenke et al., 1988). The metabolism of HCFC-123 results in the qualitative production of the same metabolites afforded by the metabolism of halothane (Harris et al., 1991; Brashear et al., 1992; Godin et al., 1993); however there have been no reports, to our knowledge, of the metabolism of HCFC-123 by humans either *in vivo* or *in vitro*. Thus, the results of this study provide the first indication that HCFC-123 is metabolized by the human.

Gruenke et al. (1988) examined the rate of TFA formation from human hepatic microsomes exposed to a concentration of 0.6% (v/v) halothane in the headspace and obtained a rate of 2.05 nmol TFA/mg protein/30 min. In the present study, 5.6 nmol TFA/mg protein/30 min was obtained in incubations containing 36% (v/v) HCFC-123 in the headspace. However, a rate of 1.7 nmol TFA/mg protein/20 min has been obtained in our laboratory from an incubation of human hepatic microsomes containing 0.6% (v/v) HCFC-123 in the headspace indicating that the two substrates are probably metabolized to the same extent *in vitro* (Godin, unpublished observation). Thus the findings reported in the present study are significant because they indicate that humans are capable of producing TFA from HCFC-123 at rates similar to those reported for halothane. The extent to which this reaction occurs in humans *in vivo* is unknown but is important in light of the hepatotoxicity associated with halothane metabolism. Trifluoroacetyl halide intermediates are produced from halothane *in vivo* and form covalent adducts with several hepatic proteins (Pohl et al., 1989; Satoh et al., 1989). Humans develop serum antibodies that have been shown to recognize these adducts (Pohl et al., 1989; Satoh et al., 1989), and their reaction has been linked to the onset of halothane-induced hepatitis. Products from the metabolism of halothane also are thought to be responsible for the hepatotoxicity observed in up to 20% of patients anesthetized with halothane (Pohl et al., 1989; Wright et al., 1975). The metabolism of HCFC-123 also affords the production of trifluoroacetylated adducts (Harris et al., 1991), but it must be pointed out that the system used in this study places an enriched cell fraction in direct contact with the chemical in solution. Whereas a PBPK prediction of liver steady-state concentrations of HCFC-123 in humans following an exposure to a 1% (v/v) atmosphere suggested that the incubations should be conducted at this same concentration, it is unlikely that humans would be exposed to a high enough concentration of HCFC-123, and for sufficient periods of time to result in a similar internal concentration. Although the amount of adduct formation resulting from brief accidental exposure

may be low, there is currently no information on the amount of adduct required to induce an immune response. In sensitized individuals therefore, hepatitis may develop after subsequent exposure to HCFC-123 or after anesthesia with halothane but this risk cannot be assessed.

REFERENCES

Brashear, W.T., M.M. Ketcha, D.L. Pollard, C.S. Godin, H.F. Leahy, P.P. Lu, E.R. Kinkead, and R.E. Wolfe. 1992. AL-TR-1992-0078, Armstrong Laboratory, Wright-Patterson Air Force Base, Ohio.

Cohen, E.N., J. R. Trudell, H.N. Edmunds, and E. Watson. 1975. *Anesthesiology* 43, 392–401.

Cousins, M.J., J.H. Sharp, G.K. Gourlay, J.F. Adams, W.D. Haynes, and R. Whitehead. 1979. *Anaesth. Intens. Care* 7, 9–24.

Ghantous, H.N., J. Fernando, A.J. Gandolfi, and K. Brendel. 1990. *Drug. Metab. Dispos.* 18, 514–518.

Godin, C.S., J.M. Drerup, and A. Vinegar. 1993. *Drug Metab. Dispos.* in press.

Gruenke, L.D., K. Konoptka, D.R. Koop, and L.A. Waskell. 1988. *J. Pharmacol. Exp. Ther.* 246, 454–459.

Harris, J.W., L.R. Pohl, J.L. Martin, and M.W. Anders. 1991. *Proc. Natl. Acad. Sci. USA* 88, 1407–1410.

Maiorino, R.M., A.J. Gandolfi, and I.G. Sipes. 1980. *J. Anal. Toxicol.* 4, 250–254.

Molina, D.M. and F.S. Rowland. 1974. *Nature* 249, 810–812.

Nakao, M., K. Fujii, H. Kinoshita, O. Yuge, and M. Morio. 1991. *Hiroshima J. Med.* Sci. 40, 23–28.

Nauck, M., D. Wolfle, and N. Katz. 1981. *Eur. J. Biochem.* 119, 657–661.

Omura, T. and R. Sato. 1964. *J. Biol. Chem.* 239, 2370–2378.

Pohl, L.R., G. Kenna, H. Satoh, D.D. Christ, and J.L. Martin. 1989. *Drug Metab. Rev.* 20, 203–217.

Rehder, K., J. Forbes, H. Alter, O. Hessler, and A. Stier. 1967. *Anesthesiology* 28, 711–715.

Satoh, H., J.M. Martin, A.H. Schulick, D.D. Christ, and J.G. Kenna. 1989. *Proc. Natl. Acad. Sci. USA* 86, 322–326.

Van Dyke, R.A. and A.J. Gandolfi 1976. *Drug Metab. Dispos.* 4, 40–44.

Wright, R., O.E. Eade, M. Chisholm, M. Hawksley, B. Lloyd, T.M. Moles, J.C. Edwards, and J.M. Gardner. 1975. *Lancet* 1, 817–820.

6.1 GENERAL PROTOCOL FOR A REPRODUCTIVE AND BEHAVIORAL TOXICITY SCREEN OF NITRITE-CONTAINING EXPLOSIVES AND PROPELLANTS ADMINISTERED IN THE DIET OR DRINKING WATER OF SPRAGUE-DAWLEY RATS

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ABSTRACT

Nitrite-containing explosives and propellants have been found to cause hemolytic anemia, methemoglobinemia, testicular atrophy, decreased spermatogenesis, and brain lesions. During restoration projects at several Army installations, measurable quantities of nitro-compounds have been found in the soil and groundwater. This protocol was developed to evaluate the potential of nitro-compounds to cause motor skill loss or produce alterations in paternal fertility, maternal pregnancy and lactation, and growth and development of offspring. This study was patterned after the Screening Information Data Set (SIDS) Program.

EXPERIMENTAL APPROACH

Reproductive Screen

A. The study begins with 12 male and 12 female rats/group to yield at least 8 pregnant females at term. Animals are assigned to the different groups by means of a computer-generated randomization stratified by body weight such that the body weights of all groups are homogeneous by statistical analysis at study initiation.

B. Group Assignments and Dose Levels

Group	Number of Animals Males	Females	Dose Level Diet or Drinking Water* (g/kg feed)	Target Dose Level (mg/kg body** wt/day)
Control	12	12	0.0	0.0
Low	12	12	TBD	TBD
Middle	12	12	TBD	TBD
High	12	12	TBD	TBD

* Concentration to be determined from a palatability test

** Estimated food consumption to be used for calculation is 25g diet/day/250g rat; water consumption is 30 mL/day/250g rat

TBD = To be determined.

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C. Test Animals

1. Species: Sprague-Dawley Derived Outbred Albino Rat [Crl:CD^{R}(SD)BR], known as Charles River CD^{R} Rat.

2. Supplier: Charles River Breeding Laboratories, Raleigh, NC.

3. Rationale: The Sprague-Dawley albino rat is a suitable species for reproduction studies because of its high fecundity rate.

4. Number and Sex: Fifty (50) female and the same number of male rats will be ordered for this study. Females will be nulliparous and nonpregnant. There will be 96 animals (48 males and 48 females) assigned to the study at the initiation of the treatment period.

5. Age and Weight: The animals will be 63 days (9 weeks) of age on the scheduled date of animal receipt (approximate weights upon arrival: 250 g for males and 175 g for females). Dosing will begin when the animals are approximately 11-weeks old (males approximately 300 g, females approximately 200 g).

D. Study Design: The test compound will be administered orally in diet or drinking water (*ad libitum*) throughout the study. The animals will be maintained on designated diet or water for two weeks prior to mating. The animals will be mated as long as 14 days, if necessary, within the appropriate treatment level. Dams will be maintained through gestation, weaning, and through a total of 90 days on study. One-half the male animals will be necropsied following mating (approximately 28 days). The remainder will be maintained through 90 days.

E. Experimental Evaluations

1. Parental Animals

a. Weight: The body weight of the male rats will be determined and recorded initially and weekly thereafter. The body weights of female rats will be recorded in the same manner until confirmation of mating. During gestation, females will be weighed on gestational days (GD) 0, 7, 14, and 20. Dams producing litters will be weighed on days 0, 4, 7, 14, and 21 postpartum, then weekly thereafter. Body weight gains will be computed.

b. Food or Water Consumption: Mean food or water consumption will be determined during the prebreeding period for both male and female rats. During pregnancy, female consumption will be measured for GD 0–7, 7–14, and 14–20. Maternal consumption will be measured for lactational days 0–7 and 7–14. Male rat consumption will be calculated weekly through sacrifice. Compound consumption (mg test material/kg body weight/day) will be calculated.

c. General Condition: The animals will be observed twice daily (a.m. and p.m.), including weekends and holidays. Clinical signs of toxicity will be recorded.

d. Mating Procedures: Animals of the parental generation will be approximately 11 weeks of age at the commencement of treatment. They will be administered treated or control food (or water) *ad libitum* for 2 weeks prior to mating; i.e., until they are approximately 13 weeks of age. The animals will then be mated on the basis of one male to one female selected randomly within each treatment group for a maximum time period of 14 days. There will be no replacement of males if mating does not occur. The observation of vaginal or dropped copulatory plugs and/or vaginal sperm will be considered to be evidence of successful mating. Females will be examined twice daily (a.m. and p.m.)

during the cohabitation period for dropped copulation plugs. Any female not exhibiting a dropped copulation plug will be examined for the presence of a copulation plug or sperm in the vaginal tract once daily (a.m.). The day a copulation plug or vaginal sperm is observed will be designated GD 0. Once a plug or vaginal sperm have been observed, the male and female from that mating pair will be individually housed. For any female which does not show evidence of successful mating after 14 days of cohabitation, the last scheduled mating day will be considered GD 0 for that female, and the animal will be treated accordingly for subsequent events. Females will be observed twice daily beginning on gd 20 for evidence of littering. The dams will be allowed to rear their young to Day 21 postpartum. On Day 21 postpartum, each litter will be weaned.

At the conclusion of the mating period, 6 males per treatment group will be sacrificed. In addition to tissues taken for histopathology, sperm motility and sperm count will be evaluated. The same parameters will be evaluated on the remaining male rats, which will continue to be treated through the conclusion of the study.

2. Progeny

   a. Mortality and Survival: All pups will be examined as soon as possible after birth to determine the number of viable and stillborn members of each litter. Survival indices will be calculated at 0, 4, 7, 14, and 21 days after birth.

   b. Body Weight and Sex Determination: All live pups will be counted, sexed, and examined grossly at birth (Postnatal day 0) and weighed individually at Days 1, 4, 7, 14, and 21 after birth. Standardization of litter sizes, 4 per sex selected randomly, when possible, will occur on Day 4. At weaning, Postnatal Day 21, one male and one female per litter will be maintained on the appropriate diet through the completion of the study. Pups maintained through the conclusion of the 90-day study will be weighed weekly.

   c. Clinical Signs: All pups will be examined for physical abnormalities at birth and observed daily throughout the postpartum period. All pups dying during this period will be necropsied when possible to investigate the cause of death.

3. Pathology

   a. All parental animals will be fasted 12 hours prior to sacrifice. All parental animals shall be subjected to a complete gross examination at necropsy. Pups sacrificed at weaning will be subjected to an external examination only. Pups maintained through the end of the study will have a complete gross necropsy. Sacrifice of all animals will occur following the completion of the 90-day study period. All of the male and female adults from the control and high-dose group will be subjected to histopathologic examination. Target organs only, identified during examination of the high-dose and control groups, will be examined from animals at the mid- and low-dose groups. Histopathologic evaluation will be conducted on the tissues specified below:

- liver
- kidneys
- brain
- pituitary
- vagina
- uterus
- ovaries
- spleen
- stomach
- duodenum
- implantation sites
- other tissues with gross lesions identified as being potentially treatment related
- testes*
- epididymides*
- scrotum
- seminal vesicles
- prostate
- bone marrow (sternal and femoral) sections
- bone marrow smear
- colon
- ileum
- corpora lutea
- thymus

*Fixed in Bouin's Fixative; sections stained with periodic acid and Schiff's (PAS); counterstained with hematoxylin.

Any of the above organs or tissues showing gross alterations will be evaluated microscopically in the mid- and low-dose groups.

b. Organs to be weighed on all parental animals will include liver, kidneys, testes, epididymides, brain, spleen, and thymus.

c. Animals Dying on Test: A complete gross necropsy and histopathologic examination will be conducted for any parental animal dying on test.

d. Clinical Pathology: A complete blood assay will be conducted on blood samples taken at sacrifice from all animals. The blood will be sampled via the posterior vena cava.

Whole Blood	**Serum**	
Hematocrit	Calcium	Total Protein
Hemoglobin	Phosphorus	Albumin
Red blood cell count	Chloride	Globulin
Platelet count	Potassium	Urea Nitrogen
Total and Differential	Creatinine	Total Bilirubin
leukocyte count	Glucose	Sodium
Methemoglobin	Magnesium	CO_2
		Alkaline phosphatase
		Alanine aminotransaminase (ALT)
		Aspartate aminotransaminase (AST)
		Thyroxine-binding globulin (TBG)
		Gamma-Glutamyl transferase (GGT)

4. Gross Necropsy: The gross examination at necropsy will include: external surfaces, all orifices, and carcass; the thoracic, abdominal, and pelvic cavities and their viscera; and cervical tissues and organs.

5. Methemoglobin Determinations: Blood taken at all sacrifices will be analyzed for methemoglobin using a cooximeter method. The amount of methemoglobin will be expressed as a percentage of the total hemoglobin present in samples. Analysis will be completed within one hour of sampling.

6. Sperm Count and Motility (performed on all parental male rats): Sperm will be removed at necropsy for count and motility analysis. Sperm removed from the epididymis will be added to a Petroff Hauserr chamber and analyzed microscopically using a videomicrography system. Count and motility analysis will be performed following the established standard operating procedure OEVM605.

BEHAVIORAL SCREEN

A. Behavioral testing will be performed on at least six rats (selected at random) from each dose level on the following schedule:

1. Pretreatment (males and females)

2. Premating (females only)

3 Postmating (males only)

4. Postpartum (females only)

5. *One-month postweaning (females only)

6. Prior to sacrifice (males, females and representative pups)

*Behavior testing will be performed at this time period only if behavioral changes have been noticed during the postpartum phase of the study.

At sacrifice, representative animals from each group will have portions of the brain removed for neurotransmitter analysis. Portions of the frontal cortex, septum, caudate nucleus, brain stem, cerebellum, hypothalamus, thalamus, hippocampus, and cerebral cortex will be sampled. The neurotransmitter analysis will be performed on isolated brain regions using HPLC methods (presently being developed).

B. Test Systems (one or more of the following tests may be performed):

1. Opto-Varimex: A computer program will collect data from infrared emitters and detectors spaced regularly along the perimeter of the 30-cm × 30-cm chamber. Capabilities will include: total distance traveled, time spent resting, time ambulatory, and time spent in "stereotypic movement," which includes rearing and circling. The number of clockwise and counterclockwise rotations will be tabulated. Exposure sessions will consist of five successive two-minute intervals conducted with the room lights off.

2. Figure Eight Maze: Each maze is connected to a computer running a simple data acquisition program. Animals are placed singly into the maze, where movement is tracked via 8 infrared emitters/detectors located along the path. The computer tallies the activity of the rat based on the number of detectors triggered. Each animal is tracked for nine sequential 10-minute sessions, for a total "observational" period of 90 minutes.

3. Startle: Animals are placed in a clear plexiglas cylinder designed to minimize movement. The animal is placed in a darkened chamber on a platform affixed to a transducer. The animal is exposed to a 70 decibel, 4000 Hz noise impulse for 120 msec. Impulses occur every 10 seconds for 50 cycles (for a total exposure time of 8 min, 20 sec). A transducer measures the vibration of the platform as the animal "flinches" at the noise. This information is tabulated and stored on the computer which controls the system.

ADMINISTRATION OF THE TEST MATERIAL

A. Route: Oral, mixed in the diet or drinking water.

B. Dose Selection: Three graduated dosage levels of the test material will be evaluated in the study. The test substance will be administered in the diet or drinking water at a constant concentration basis. It is anticipated that at the higher dosage level(s) some toxicological or pharmacological effect(s) will be observed, and that at the lower dosage level(s) no treatment-related effects will be seen. A concurrent control group will be used in this study. Dosage levels will be expressed in terms of mg/kg body weight/day.

***REPRODUCTIVE AND GENERAL TOXICITY PARAMETERS RECORDED AND/OR* MEASURED**

A. Individual Maternal Data

1. Identification number.
2. Age at beginning of study.
3. Age at death and manner of death.
4. Weekly body weights prior to mating and gestational and lactational body weights taken thereafter.
5. Prebreed, gestational, and lactational food consumption.
6. Hematology, clinical chemistry, and methemoglobin levels in whole blood.
7. Male rat (by identification number) used for mating.
8. Gestational length in days.
9. Total number of offspring per litter.
10. Number and percent of live and dead offspring.
11. General condition of offspring and mother through weaning.
12. Organ weights.
13. Histopathology.

B. Summary of Maternal Data

1. Mean periodic maternal body weights and weight gains.

2. Compound consumption (expressed as mg/kg/day).

3. Hematology, clinical chemistry, and methemoglobin levels in whole blood.

4. Survival indices.

5. Mean litter size.

6. Mean number of live and dead offspring.

7. Number and percent of mothers showing treatment-related behavioral abnormalities in nesting and nursing.

8. Mating index (%) = $\frac{\text{Number of females plug or sperm positive}}{\text{Number of females paired.}} \times 100$

9. Fertility index (%) = $\frac{\text{Number of females pregnant}}{\text{Number of females plug or sperm positive.}} \times 100$

10. Organ Weights.

11. Histopathology.

C. Individual Paternal Data

1. Identification number.

2. Age at beginning of study .

3. Age at death and manner of death.

4. Weekly body weights through mating.

5. Hematology, clinical chemistry, and methemoglobin levels in whole blood.

D. Summary of Paternal Data

1. Mean body weights, weight gains, and survival indices.

2. Compound consumption (expressed as mg/kg/day).

3. Hematology, clinical chemistry, and methemoglobin levels in whole blood.

4. Mating index (%) = $\frac{\text{Number of males impregnating females}}{\text{Number of males paired.}} \times 100$

5. Fertility index (%) = $\frac{\text{Number of males siring litters}}{\text{Number of males impregnating females.}} \times 100$

6. Organ weights.

7. Histopathology.

E. Summary of Litter Data

1. Total litter size.

2. Number and percent of stillborn.

3. Number and percent of live births.

4. Periodic viability counts.

5. Periodic body weights by sex per litter from day 1 of life through weaning (taken on days 1, 4, 7, 14, and 21 of lactation by individual pup), weekly thereafter.

6. Pup survival indices (also expressed as percentages).

7. Sex ratio.

8. Indices:

$$\text{Gestational Index} = \frac{\text{Number of females with live litter}}{\text{Number of females pregnant.}}$$

$$\text{Live birth index} = \frac{\text{Number of live pups at birth}}{\text{Total number of pups born.}}$$

$$\text{4-Day Survival index} = \frac{\text{Number of pups surviving 4 days}}{\text{Total number of live pups at birth.}}$$

$$\text{7-Day Survival index} = \frac{\text{Number of pups surviving 7 days}}{\text{Total number of live pups at 4 days (postcull).}}$$

$$\text{14-Day Survival index} = \frac{\text{Number of pups surviving 14 days}}{\text{Total number of live pups at 7 days.}}$$

$$\text{21-Day Survival index} = \frac{\text{Number of pups surviving 21 days}}{\text{Total number of live pups at 14 days.}}$$

$$\text{Lactation index} = \frac{\text{Number of pups surviving 21 days}}{\text{Total number of live pups at 4 days (postcull).}}$$

STATISTICAL ANALYSIS

The unit of comparison will be the male or the pregnant female (or the litter). Results of the quantitative continuous variables (e.g., parental and pup body weights) will be intercompared for the three treatment groups and one control group by the use of Levene's test of equal variances, and analysis

of variance (ANOVA). When Levene's test indicates homogenous variances and the ANOVA is significant, a Bonferroni t-test will be used for pairwise comparisons. When Levene's test indicates heterogeneous variances, all groups will be compared by an ANOVA for unequal variances or an appropriate transformation will be done.

Nonparametric data will be statistically evaluated using the Kruskal-Wallis test followed by the Mann-Whitney U–test for pairwise comparisons when appropriate. Frequency data (such as the various indices) will be compared using the Fisher's Exact Test. For all statistical tests, the fiducial limit of 0.05 (two-tailed) will be used as the criterion for significance.

6.2 REPRODUCTIVE TOXICITY SCREEN OF 1,3,5-TRINITROBENZENE ADMINISTERED IN THE DIET OF SPRAGUE-DAWLEY RATS

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ABSTRACT

Several Army installations targeted for restoration have measurable quantities of 1,3,5-trinitrobenzene (TNB) in the soil and groundwater. As part of the process to develop environmental and health effects criteria for restoration, a modified Screening Information Data Set (SIDS) reproductive study was performed. Male and female Sprague-Dawley rats received a diet containing approximately 300, 150, or 30 mg TNB/kg diet. Mating occurred following 14 days of treatment. All dams, one-half the males, and representative pups were maintained for a total of 90 days of treatment. No mortality occurred during the study; however, a decrease in mean body weights was noted in both sexes of high-dose rats. A dose-related effect was noted in measurements of sperm function/activity. Sperm depletion and degeneration of the seminiferous tubules were noted histopathologically. Methemoglobinemia and splenic hemosiderosis were common findings in the high- and mid-dose levels of both sexes at necropsy. No adverse effects were noted in mating or fertility indices. No significant treatment-related differences were found in length of gestation, sex ratio, gestation index, or mean number of pups per litter.

INTRODUCTION

Several Army installations targeted for restoration have measurable quantities of 1,3,5-trinitrobenzene (TNB) in the soil and groundwater. 1,3,5-Trinitrobenzene is a dimorphic crystalline solid that is easily dissolved in organic solvents (Fedoroff et al., 1962). It is produced during the nitration step of trinitrotoluene synthesis as a result of methyl group oxidation (Budavari et al., 1989). It is primarily used as an explosive, but has also had limited use in the vulcanization of rubber (Barnhart, 1981). 1,3,5-Trinitrobenzene and a similar compound, 1,3-dinitrobenzene (DNB), are used to produce plastics, herbicides, and paints and can enter domestic drinking water reservoirs via domestic effluent (Ryon et al., 1984; U.S. EPA, 1989). These compounds are not readily biodegradable and have a tendency to leach out into the groundwater near production or disposal sites.

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Fitzgerald et al. (1992) reported acute toxicity and irritancy data for TNB. The acute oral toxicity of TNB suspended in corn oil was reported as 298 and 275 mg/kg in male and female rats, and >900 and 702 mg/kg in male and female mice, respectively. No deaths occurred when neat TNB was in contact with rabbit skin for 24 h (2 g/kg limit test). 1,3,5-Trinitrobenzene was found to be a mild skin sensitizer in guinea pigs, but did not cause acute irritation on rabbit skin. When applied as a powder, the treated eyes of all test rabbits were scored "severe" for redness, chemosis, and opacity through 96 h posttreatment. 1,3,5-Trinitrobenzene caused irreversible damage to ocular tissue and is considered to be corrosive.

Munition workers exposed to 2,4,6-trinitrotoluene (TNT) have developed skin irritation, liver damage, and anemia (Hathaway, 1977; Morton et al., 1976; Stewart et al., 1945). Animal studies have shown that oral treatment with structurally similar DNB or TNT causes anemia, increases methemoglobin concentration, and produces hypertrophy of the liver and spleen. Degeneration of the germinal epithelial lining of the seminiferous tubules also occurs, resulting in decreased spermatogenesis (Cody et al., 1981; Levine et al., 1983, 1984; Furedi et al., 1984a,b; Linder et al., 1986).

A range-finding reproduction study in which Sprague-Dawley rats received TNB-treated diet at concentrations of approximately 800, 400, and 67 mg TNB/kg diet resulted in testicular atrophy and sperm depletion in male rats receiving the two highest concentrations (Kinkead et al., 1994). Head tilt and loss of equilibrium occurred in the high-dose female rats during the postpartum period, and both sexes had brain lesions in the olivary region of the medulla at the conclusion of the range-finding study. Head tilt and loss of equilibrium (Linder et al., 1990), as well as brain and brain stem lesions (Philbert et al., 1987), have also been reported in rats following treatment with DNB.

MATERIALS AND METHODS

Test Agent and Doses

The TNB/diet mixture was provided by the U.S. Army through a contract with the Environmental Protection Agency. Pertinent chemical and physical properties of the test compound are listed below:

1,3,5-Trinitrobenzene (TNB)

Synonyms:	Trinitrobenzene
	Benzite
CAS #:	99-35-4
Empirical Formula:	$C_6H_3N_3O_6$
Formula Weight:	213.11
Vapor Pressure	3.2×10^{-6} mmHg at 20 °C

The TNB was administered orally by mixing the test material into ground Purina Formulab #5002 (Ralston Purina, St. Louis, MO) certified rodent diet meal. Mean concentrations (and ranges) for each test concentration were 287 (278–305), 147 (138–158), and 30.7 (29.4–31.9) mg TNB/kg diet (five batches per treatment level).

Group Assignments and Dose Levels

Group	Number of Animals		Target Dose Level of TNB (mg/kg diet)	Target Dose Level of TNB (mg/kg body weight/day)
	Male	Female		
Control	18	12	0.0	0.0
Low	18	12	30.0	1.8
Middle	18	12	150.0	9.0
High	18	12	300.0	18.0

[a]Assume 500 g rat consumes 30 g feed/day.

Study Design and Experimental Evaluations

The test compound was administered orally in the diet. The study design, experimental evaluations, and animal assessments for this study are detailed in section 6.1 of this annual report. A technical report entitled "Reproductive Toxicity Screen of 1,3,5-Trinitrobenzene Administered in the Diet of Sprague-Dawley Rats" by Kinkead, Wolfe, Flemming, Caldwell, Miller, and Marit (AL/OE-TR-1994-0144; WRAIR-TR-1994-0016) contains details of assessment data and figures not included in this annual report.

RESULTS

Food Consumption and Calculated Dose

Food consumption decreased significantly in the high- and mid-dose groups of both sexes when treatment began. Food consumption returned to pretreatment levels after four days. Food consumption of the female rats increased during the postpartum (lactation) period compared to the premating and gestation periods. Male rats consumed approximately 30 g diet/day, which resulted in a dose of approximately 19, 9, and 2 mg TNB/kg/day in the high-, mid-, and low-dose groups, respectively. Female rats consumed an average of 30 g diet/day, which converts to approximately 29, 14, and 3 mg TNB/kg/day for the high-dose, mid-dose, and low-dose groups, respectively.

General Toxicity

No mortality occurred in the parental animals during the study. Mean body weights of the high-dose male rats were significantly less than the mean body weights of the control group beginning on Day 21 and continuing through the 90-day treatment period. Male rat groups maintained posttreatment; all showed increased weight gain when returned to untreated diet for the final two months. However, the mean body weight of the high-dose group, although not significantly different, remained lower than the control group. Mean body weights of the high-dose female rats were slightly less than the control group throughout the study; however, the difference was statistically significant only at Lactation Day 0.

No clinical signs of motor skill loss were noted during the study. This was confirmed by the Opto-Varimex tests in which no differences in locomotor skills were noted in either treated or control animals.

Six male rats per group were necropsied following the mating period (28 days of treatment), after 90 days of treatment, and following a two-month recovery period. No treatment-related differences were noted in absolute or relative organ weights in male rats at any of the evaluation periods. An increase in absolute and relative spleen weights was noted in the high-dose female rats necropsied following 90 days of treatment. Relative liver weights were increased in the high-dose group and relative kidney weights were increased in both the high- and mid-dose female groups (Table 6.2-1).

TABLE 6.2-1. ABSOLUTE AND RELATIVE ORGAN WEIGHTS OF FEMALE RATS TREATED WITH TNB FOR 90 DAYS

Organ	TNB Dose			
	Control	Low	Medium	High
Liver	9.87 ± 0.32	10.21 ± 0.48	10.05 ± 0.24	9.91 ± 0.35
Ratio[b]	3.06 ± 0.06	3.03 ± 0.08	3.22 ± 0.07	3.34 ± 0.07[c]
Spleen	0.60 ± 0.01	0.63 ± 0.03	0.66 ± 0.02	0.69 ± 0.02[c]
Ratio	0.19 ± < 0.01	0.19 ± 0.01	0.21 ± 0.01	0.23 ± 0.01[d]
Kidneys	2.16 ± 0.06	2.26 ± 0.08	2.27 ± 0.06	2.17 ± 0.06
Ratio	0.67 ± 0.01	0.67 ± 0.01	0.73 ± 0.01[c]	0.73 ± 0.01[c]
Brain	2.01 ± 0.02	1.98 ± 0.09	1.97 ± 0.03	1.95 ± 0.03
Ratio	0.63 ± 0.02	0.59 ± 0.02	0.63 ± 0.01	0.66 ± 0.01
Thymus	0.34 ± 0.02	0.42 ± 0.03	0.37 ± 0.03	0.37 ± 0.02
Ratio	0.11 ± < 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
Body Wt.	321.8 ± 7.3	336.4 ± 10.2	313.4 ± 8.0	296.4 ± 6.1

[a]Mean ± SEM, N = 12.
[b]Organ weight/body weight × 100.
[c]Statistically different from control at $p < 0.01$.
[d]Statistically different from control at $p < 0.05$.

High-dose male rats, sacrificed following mating (28 days) and after 90 days of treatment, showed adverse effects for most measurements of sperm function/activity (Table 6.2-2). The number and concentration of motile cells were greatly reduced in the high-dose group. The percent of cells traveling in a circular pattern was reduced in both the high-dose and the mid-dose groups after 28 and 90 days of treatment. Sperm function/activity evaluated in the TNB-treated rats maintained on control diet for two months was similar to the sperm function of control rats (Table 6.2-2).

TABLE 6.2-2. SPERM EVALUATIONS FROM RATS ADMINISTERED TNB IN DIET

Parameter	TNB Dose			
	Control (N = 6)	Low (N = 6)	Mid (N = 6)	High (N = 6)
28 Days of Treatment				
Mean (± SEM) Number Motile Cells Analyzed	406.5 ± 26.1	383.2 ± 36.9	329.7 ± 28.8	256.5 ± 3.1[a]
Concentration Motile (million/mL)	1.74	1.12	1.05	0.83[a]
Mean (± SEM) Number of Cells Traveling in a Circular Pattern	87.5 ± 8.0	90.8 ± 4.2	73.0 ± 4.2[b]	52.3 ± 6.9[a]
Percent Cells Traveling in a Circular Pattern	16.0	17.8	15.9	11.3[b]
Percent in Circular Pattern Compared to Total Motile Cells	21.5	24.4	22.6	20.4
90 Days of Treatment				
Mean (± SEM) Number Motile Cells Analyzed	497.7 ± 47.7	504.8 ± 59.3	361.5 ± 67.8	229.0 ± 5.8[a]
Concentration Motile (million/mL)	1.69	1.67	1.37	0.78[a]
Mean Number of Cells Traveling in a Circular Pattern	84.3 ± 6.0	87.8 ± 10.9	60.8 ± 11.3[b]	55.6 ± 13.9[a]
Percent Cells Traveling in a Circular Pattern	11.7	11.7	10.0[b]	10.0[b]
Percent in Circular Pattern Compared to Total Motile Cells	17.3	17.4	16.9	24.5[b]
2 Months Posttreatment				
Mean (± SEM) Number Motile Cells Analyzed	380.8 ± 34.4	321.2 ± 55.2	375.3 ± 13.5	343.2 ± 29.6
Concentration Motile (million/mL)	1.19	1.03	1.20	1.03
Mean Number of Cells Traveling in a Circular Pattern	87.3 ± 5.9	68.3 ± 13.2	86.0 ± 3.6	79.5 ± 6.8
Percent Cells Traveling in a Circular Pattern	19.1	13.3	18.3	17.0
Percent in Circular Pattern Compared to Total Motile Cells	23.6	21.3	23.2	23.2

[a]Different from control at $p < 0.01$.
[b]Different from control at $p < 0.05$.

Methemoglobin concentration was significantly ($p < 0.01$) increased in the high- and mid-dose rats of both sexes following either 28 or 90 days of treatment (data not shown). A significant decrease in percent neutrophils and significant increase in lymphocytes was noted in the high- and mid-dose female rats after 90 days. The high-dose female rats also showed a significant decrease in hemoglobin values. Male rats which were maintained posttreatment on control diet had methemoglobin values similar to those of the control group.

After 28 days of treatment, calcium levels were increased in the high-dose male rat group and alkaline phosphatase values were low in the low-dose male rat group (data not shown). There were no abnormalities noted in the selected clinical chemistry parameters measured in male rats following 90 days of treatment or after the 2-month recovery period. Cholesterol values were significantly increased in the high-dose female rat group and LDH values were significantly decreased in the mid-dose female rat group at the conclusion of the study.

At necropsy all animals used in the study were in good general condition. Dilation of the uterus by clear fluid was noticed in two rats from both the high- and mid-dose groups, and in one rat from the low-dose group. This finding was not observed in any of the control female rats. Discoloration of the kidneys was observed exclusively in males involving two to three animals per group, including controls. Other nontreatment-related gross observations included testicular atrophy, splenomegaly, and discoloration of the thymus and liver (each observed only once). Gross examination of the pups revealed foci of tan discoloration in the liver of one animal from the control group.

Histopathology

Observed histopathologic lesions of statistical, clinical, or pathologic significance were limited to the spleen, kidney, and testes (Tables 6.2-3 through 6.2-6). After 28 days of treatment, severity and incidence of splenic hemosiderosis was significantly increased in the mid- ($p < 0.05$) and high-dose ($p < 0.01$) male rats. At 90 days, the severity of splenic hemosiderosis was increased ($p < 0.01$) in the high-dose males when compared to controls. The severity of splenic hemosiderosis was also significantly ($p < 0.01$) increased in the mid- and high-dose female rats examined after 90 days.

TABLE 6.2-3. INCIDENCE SUMMARY OF SELECTED MICROSCOPIC LESIONS OF MALE RATS

Following 28 Days of Treatment with TNB

Organ/Lesion	TNB DOSE			
	Control	Low	Medium	High
Kidney (N)	6	6	6	6
Lymphoid infiltrates	100	67	17[c]	50
(severity)[a]	1.0	0.7	0.2[c]	0.8
Hyaline droplets	100	100	100	100
(severity)	2.8	2.7	2.3	2.2
Spleen (N)	6	6	6	6
Hemosiderosis	0	0	50[c]	100[b]
(severity)	0.0	0.0	0.5[c]	1.0[b]
Testes (N)	6	6	6	6
Tubular degeneration	17	0	0	0
(severity)	0.3	0.0	0.0	0.0
Epididymis (N)	6	6	6	6
Atypical sperm[d]	0	0	0	0

[a]Mean grades of severity based on 0 = Normal; 1 = Minimal; 2 = Mild; 3 = Moderate; 4 = Marked; 5 = Severe.
[b]Statistically different from control at $p < 0.01$.
[c]Statistically different from control at $p < 0.05$.
[d]Signifies lesion present only, severity not graded.

TABLE 6.2-4. INCIDENCE SUMMARY OF SELECTED MICROSCOPIC LESIONS OF MALE RATS FOLLOWING 90 DAYS OF TREATMENT WITH TNB

	TNB Dose			
Organ/Lesion	Control	Low	Medium	High
Kidney (N)	6	6	6	6
Lymphoid infiltrates	67	100	100	100
(severity)[a]	0.7	1.0	1.0	1.3[c]
Hyaline droplets	33	100[b]	100[b]	100[b]
(severity)	0.5	1.2	2.2[c]	3.0[c]
Spleen (N)	6	6	6	6
Hemosiderosis	100	100	100	100
(severity)	1.3	1.3	1.5	2.2[b]
Testes (N)	6	6	6	6
Tubular degeneration	0	0	0	50[c]
(severity)	0.0	0.0	0.0	1.0[b]
Epididymis (N)	6	6	6	6
Atypical sperm[d]	0	0	0	50[c]

[a]Mean grades of severity based on 0 = Normal; 1 = Minimal; 2 = Mild; 3 = Moderate; 4 = Marked; 5 = Severe.
[b]Statistically different from control at $p < 0.01$.
[c]Statistically different from control at $p < 0.05$.
[d]Signifies lesion present only, severity not graded.

TABLE 6.2-5. INCIDENCE SUMMARY OF SELECTED MICROSCOPIC LESIONS OF FEMALE RATS FOLLOWING 90 DAYS OF TREATMENT WITH TNB

	TNB Dose			
Organ/Lesion	Control	Low	Medium	High
Kidney (N)	12	12	12	12
Lymphoid infiltrates	42	58	17	58
(severity)[a]	0.4	0.6	0.2	0.8
Hyaline droplets	0	0	33	66[b]
(severity)	0.0	0.0	0.3	0.8[b]
Spleen (N)	12	12	12	12
Hemosiderosis	100	100	100	100
(severity)	1.2	1.3	2.4[b]	3.0[b]

[a]Mean grades of severity based on 0 = Normal; 1 = Minimal; 2 = Mild; 3 = Moderate; 4 = Marked; 5 = Severe.
[b]Statistically different from control at $p < 0.01$.

TABLE 6.2-6. INCIDENCE SUMMARY OF SELECTED MICROSCOPIC LESIONS OF MALE RATS TWO MONTHS FOLLOWING TREATMENT WITH TNB

	TNB Dose			
Organ/Lesion	Control	Low	Medium	High
Kidney (N)	6	6	6	6
Lymphoid infiltrates	100	100	83	100
(severity)[a]	1.3	1.0	0.8	1.3
Hyaline droplets	100	100	83	83
(severity)	1.0	1.0	0.8	0.8
Spleen (N)	6	6	6	6
Hemosiderosis	83	100	100	100
(severity)	1.2	1.5	1.8	1.8
Testes (N)	6	6	6	5
Tubular degeneration	0	0	0	40[c]
(severity)	0.0	0.0	0.0	0.6[c]
Epididymis (N)	6	6	6	5
Atypical sperm[d]	0	0	17	20

[a]Mean grades of severity based on 0 = Normal; 1 = Minimal; 2 = Mild; 3 = Moderate; 4 = Marked; 5 = Severe.
[b]Statistically different from control at $p < 0.01$.
[c]Statistically different from control at $p < 0.05$.
[d]Signifies lesion present only, severity not graded.

There was a significant decrease in the incidence and severity of renal interstitial lymphoid infiltrates in the mid-dose male rats examined after 28 days of treatment. At 90 days, there was increased incidence and severity of hyaline droplets in all TNB-treated male rats. There was also an increased ($p < 0.05$) severity in renal lymphoid filtrates in the high-dose male rats. Severity and incidence of hyaline droplet change was also significantly increased ($p < 0.01$) in the high-dose female rats after 90 days.

There was a significant increase in the incidence and severity of testicular seminiferous tubular degeneration after 90 days of treatment, and after 2-months recovery, that was not noted after 28 days of treatment. There was also a corresponding increased ($p < 0.05$) incidence of sperm abnormality seen in the epididymides of the high-dose rats following 90 days of treatment. However, there was recovery after two months, as both incidence and severity of the lesions were reduced.

Reproductive Indices

The treatment showed no adverse effects on mating as 100% of the animals mated (Table 6.2-7). The fertility index was 92% in the high-dose group and 100% in the other treated and control groups. No significant treatment-related differences were noted in length of gestation, sex ratio, gestation index, or mean number of offspring per litter. During the 21-day lactation phase, the mean body weights of the TNB-treated pups, both male and female, were significantly lower than the control group pups except at 14 days, when the mid- and low-dose pup weights were equivalent to controls. Because no differences in mean body weights were noted between sexes of pups during the 21-day period, the sexes were combined for statistical analyses.

TABLE 6.2-7. LITTER DATA FOR RATS TREATED WITH TNB

	TNB Dose			
	Control	**Low**	**Medium**	**High**
No. of Mated Pairs	12	12	12	12
No. of Copulated Pairs	12	12	12	11
No. of Dams with Pups Born	12	12	12	11
No. of Dams with Pups Alive	12	12	12	11
Fertility Index (%)	100.0	100.0	100.0	91.7
Gestation Index (%)[a]	100.0	100.0	100.0	100.0
Live Birth Index (%)[b]	97.5	100.0	97.8	98.1
4-Day Survival Index (%)	98.7	100.0	100.0	98.1
7-Day Survival Index (%)	100.0	98.9	100.0	100.0
14-Day Survival Index (%)	100.0	100.0	100.0	100.0
21-Day Survival Index (%)	100.0	100.0	100.0	100.0
Lactation Index (%)[c]	100.0	98.9	100.0	100.0

[a] $\frac{\text{Number of females with live litters}}{\text{Number of females pregnant}}$

[b] $\frac{\text{Number of live pups at birth}}{\text{Total number of pups born}}$

[c] $\frac{\text{Number of pups surviving 21 days}}{\text{Number of pups surviving 4 days}}$

DISCUSSION

Administering TNB in the diet of Sprague-Dawley rats at calculated dose levels of approximately 19, 9, and 2, or 29, 14, and 3 mg TNB/kg body weight/day of males and females, respectively, produced no adverse effects on reproductive performance or litter parameters. Treatment-related decreases in mean body weights of pups (as great as 15% in the high-dose group) were observed during the 21-day postpartum period.

Anemia and increased methemoglobin production are common characteristics of nitrate poisoning. Although anemia was not noted in this study, the increased incidence and/or severity of splenic hemosiderosis suggests an increased removal (phagocytosis) of erythrocytes. The change in leukocyte parameters in the mid- and high-dose female rats, as well as alteration in cholesterol values in the high-dose females, is not believed to be biologically significant.

Brain lesions or motor skills loss were not noted following treatment at these dose levels as was noted following treatment with DNB or higher doses of TNB (Linder et al., 1990; Kinkead et al., 1994). However, sperm depletion and degeneration of the seminiferous tubules at the high-dose level during the treatment period was clearly a treatment-related effect, typical of nitrate toxicity (Linder et al., 1986, 1990; Cody et al., 1981). Sperm loss and degeneration had no effect on mating as the fertility index of the TNB-treated rats was > 90%. Full recovery from testicular lesions did not occur within 60 days after removal from TNB-treated diet; however, sperm concentration did return to control levels.

In summary, TNB has been shown to disrupt spermatogenic activity at doses as low as 9 mg TNB/kg/day, but the testicular effects are not nearly as potent as with DNB. Although the sperm concentration in TNB-treated rats was reduced, fertility of the male rats appeared to be unaffected.

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REFERENCES

Barnhart, R.R. 1981. Rubber Compounding. In: *Kirk-Othmer Encyclopedia of Chemical Technology*, 3rd Ed., Vol. 20. (A. Stannden, Ed.) John Wiley and Sons, New York, NY. pp. 393–468.

Budavari, S., M.J. O'Neil, and A. Smith, Eds. 1989. In: *The Merck Index*. Merck & Co., Inc., Rahway, NJ. pp. 1530.

Cody, T.E., S. Witherup, L. Hastings, K. Stemmer, and R.T. Christian. 1981. 1,3-Dinitrobenzene: Toxic effect *in vivo* and *in vitro*. *J. Toxicol. Environ. Health* 7(5):829–847.

Fedoroff, B.T., O.E. Sheffield, E.F. Reese, and G.D. Clift, Eds. 1962. In: *Encyclopedia of Explosives and Related Items*. PATR 2700, Vol. 2. Picatinny Arsenal, Dover, NJ. pp. B48–B49.

Fitzgerald, C.B., N. DiGiulio, L.S. Desai, and G. Reddy. 1992. Acute toxicological evaluation of 1,3,5-Trinitrobenzene. *Acute Toxicity Data* 1(3):169–170.

Furedi, E.M., B.S. Levine, D.E. Gordon, V.S. Rac, and P.M. Lish. 1984a. Determination of the chronic mammalian toxicologic effects of TNT (Twenty-four month chronic toxicity/carcinogenicity study of trinitrotoluene (TNT) in the Fischer 344 rat). Final Report - Phase III, Vol. 1. IIT Research Institute, Project No. L6116, Study No. 11, Chicago, IL. DAMD17-79-C-9120. AD-A168 754.

Furedi, E.M., B.S. Levine, J.W. Sagartz, V.S. Rac, and P.M. Lish. 1984b. Determination of the chronic mammalian toxicologic effects of TNT (Twenty-four month chronic toxicity/carcinogenicity study of trinitrotoluene (TNT) in the $B6C3F_1$ hybrid mouse). Final Report - Phase IV, Vol. 1. IIT Research Institute, Project No. L6116, Study No. 11, Chicago, IL. DAMD17-79-C-9120. AD-A168 754.

Hathaway, J.A. 1977. Trinitrotoluene: A review of reported dose-related effects providing documentation for a workplace standard. *J. Occup. Med.* 19(5):341–345.

Kinkead, E.R., R.E. Wolfe, S.A. Salins, C.D. Flemming, D.J. Caldwell, C.R. Miller, and J.R. Latendresse. 1994. Range-Finding Study for a Reproductive Assessment of 1,3,5-Trinitrobenzene Administered in the Diet of Sprague-Dawley Rats. AL-TR-1994-072/WRAIR-TR-1994-0006. Wright-Patterson Air Force Base, OH: Armstrong Laboratory and Walter Reed Army Institute of Research.

Levine, B.S., J.H. Rust, J.M. Burns, and P.M. Lish. 1983. Determination of the chronic mammalian toxicological effects of TNT (Twenty-six week subchronic oral toxicity study of trinitrotoluene (TNT) in the beagle dog). Phase II, Final Report, IIT Research Institute, Report No. L6116, Study No. 5, Chicago, IL. DAMD 17-79-C-9120, AD-A157 082.

Levine, B.S., E.M. Furedi, D.E. Gordon, P.M. Lish, and J.J. Barkely. 1984. Subchronic toxicity of trinitrotoluene in Fischer 344 rats. *Toxicology* 32:253–265.

Linder, R.E., R.A. Hess, and L.F. Strader. 1986. Testicular toxicity and infertility in male rats treated with 1,3-dinitrobenzene. *J. Tox. and Environ. Health* 19:477–489.

Linder, R.E., L.F. Strader, R.R. Barbee, G.L. Rehnberg, and S.D. Perreault. 1990. Reproductive toxicity of a single dose of 1,3-dinitrobenzene in two ages of young adult male rats. *Fundam. Appl. Tox.* 14:284–298.

Morton, A.R., M.V. Ranadive, and J.A. Hathaway. 1976. Biological effects of trinitrotoluene from exposure below the threshold limit value. *Am. Ind. Hyg. Assoc. J.* 37(1):56–60.

Philbert, M.A., A.J. Gray, and T.A. Connors. 1987. Preliminary investigations into the involvement of the intestinal microfluora in CNS toxicity induced by 1,3-dinitrobenzene in male F-344 rats. *Tox Letters* 38:307–314.

Ryon, M.G., B.C. Pal, S.S. Talmage, and R.H. Ross. 1984. Database assessment of the health and environmental effects of munition production waste products. Final Report. ORNL-6018. (NTIS DE84-016512). Oak Ridge National Laboratory, Oak Ridge, TN.

Stewart, A., L.T. Witts, G. Higgins, et al. 1945. Some early effects of exposure to trinitrotoluene. *Br. J. Ind. Med.* 2:74–82. (Cited in Hathaway, 1977)

U.S. EPA. 1989. Health and Environmental Effects Profile for 1,3,5-Trinitrobenzene. Environmental Criteria and Assessment Office, Office of Health and Environmental Assessment, Cincinnati, OH, ECAO-CIN-G071.

6.3 REPRODUCTIVE TOXICITY SCREEN OF LIQUID PROPELLANT XM46 ADMINISTERED IN THE DRINKING WATER OF SPRAGUE-DAWLEY RATS

E.R. Kinkead, R.E. Wolfe, S.A. Salins, C.D. Flemming, H.F. Leahy, D.J. Caldwell[1], C.R. Miller[1], and G.B. Marit[2]

ABSTRACT

Liquid propellant (XM46) is being considered as a replacement for solid propellants, both as part of a regenerative injection gun system and as a working fluid in an electrothermal gun system. The XM46 formulation contains hydroxylammonium nitrate, triethanolammonium nitrate, and water. Male and female Sprague-Dawley rats received XM46 in drinking water containing 2.0, 1.0, 0.2, or 0.0 g XM46/L throughout 90 days of study. Mating occurred following 14 days of treatment. One half the male rats per group were necropsied after 28 days of treatment and the remaining males and all dams were necropsied following 90 days. No mortality occurred in any of the parental animals during the study. The study did not produce any adverse effects on reproduction or litter parameters. Hemolytic anemia and methemoglobinemia were common findings in both sexes of rats. Splenomegaly was found in both sexes; in male rats as early as 28 days. Exposures via drinking water containing XM46 for 90 days did not result in any decrease in reproductive performance in male or female rats, but it did result in clinical signs of hemolytic anemia at calculated doses as low as 17 mg/kg/day.

INTRODUCTION

Liquid propellant 1846 (XM46) is a developmental propellant for the next generation Advanced Field Artillery System. It will be used as part of the regenerative injection gun system instead of powder charges for the 155-mm cannon and as a working fluid in an electrothermal gun system. The components of XM46 (hydroxylammonium nitrate [HAN] and triethanolammonium nitrate) are both strongly reducing and oxidizing agents and can react with many organic and inorganic materials (Jet Propulsion Laboratory, 1989). Impurities are generated by reaction of XM46 with components of the gun systems (e.g., metals), manufacturing intermediates, or materials introduced by improper handling after production. Decomposition leads to the formation of nitric acid and ammonium nitrate with subsequent destabilization of the propellant mixture (Klein et al., 1991; Hansen et al., 1990).

Individuals working with XM46 have reported burning sensations and lesions within 24 h following exposure. Sensitization has been reported in one worker that handled HAN (Parmer et al., 1991). XM46 is a moderate to severe skin irritant and may cause a burning sensation immediately upon contact. If the

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material is not flushed, erythema will occur on contaminated areas within several hours, followed by severe dermatitis within 24 h (Weller et al., 1989).

When applied to guinea pig skin, XM46 proved to be a strong skin sensitizer. The sensitization response to XM46 may be associated with hydroxylamine, a potent skin sensitizer (Gross, 1985) and to triethylamine, also reported to be a skin sensitizer (Grosselin et al., 1984). Single- and repeat-dose dermal studies on animals have shown that XM46 and HAN penetrate the skin to produce systemic effects. A 21-day repeated dermal application of XM46 and HAN to rabbit skin produced erythrocyte destruction, Heinz body formation, anemia, spleen enlargement, and dermal necrosis (Asaki et al., 1982). Rinsing the skin within four hours of exposure effectively reduces the severity of the skin lesions (Witt et al., 1992).

XM46 is also a strong eye irritant, producing iritis, chemosis, and corneal opacity which lasts for up to one week. Washing the eye 30 seconds after application eliminated the corneal opacity and reduced the duration and severity of the remaining effects. Washing at 10 minutes postapplication further reduced the symptoms, but signs of ocular irritation were still present (Justus and Korte, 1988).

Inhalation of XM46 vapor is not a hazard since the vapor decomposes almost entirely to water. Inhalation of HAN aerosol, however, may produce respiratory irritation, blood dyscrasia, and elevated methemoglobin (Snodgrass et al., 1985). Extensive genotoxicity studies have been negative (Jagannath, 1979; Balter et al., 1982; Bakke, 1990; Rudd and Lee, 1990; Blachman, 1990) and XM46 is classified as a nonmutagen.

The purpose of this study was to evaluate the potential of XM46 to produce alterations in paternal and/or maternal fertility, maternal gestation and lactation, and growth and development of offspring of Sprague-Dawley rats. The XM46 was administered to rats via drinking water.

Test Compound

The XM46 formulation is a mixture of hydroxylammonium nitrate (61%), triethanolammonium nitrate (19%), and water (20%). The compound is a eutectic salt and does not exist as an aqueous solution. The XM46 formulation is acidic and has a density of 1.42 g/mL at 20 °C.

Group Assignments and Dose Levels

Group	Number of Animals		Dose Level of XM46 (mg/L drinking water)	Target Dose Level of XM46 (mg/kg body wt/day)[a]
	Males	Females		
Control	12	12	0.0	0.0
Low	12	12	200.0	12.0
Middle	12	12	1000.0	60.0
High	12	12	2000.0	120.0

[a]Assumed daily water consumption of 30 mL per 500 g rat.

Study Design and Experimental Evaluations

The test compound was administered orally in drinking water (*ad libitum*). The study design, experimental evaluations and animal assessments for this study are detailed in Section 6.1 of this Annual Report. A technical report entitled "Reproductive Toxicity Screen of Liquid Propellant 1846 (XM46) Administered in the Drinking Water of Sprague-Dawley Rats" by Kinkead, Wolfe, Salins, Flemming, Miller, Caldwell, and Marit (AL/OE-TR-1994-0101; WRAIR/TR-94-0008) contains details of assessment data and figures not included in this Annual Report.

RESULTS

General Toxicity

No mortality occurred in parental rats during the study. No treatment-related differences were noted in mean body weights of treated rats when compared to their respective control group. Water consumption (not shown) decreased significantly in both sexes of treated rats when treatment began and continued to be significantly less than controls throughout the 90-day study. For the duration of the study male rats consumed approximately 30, 30, and 37 mL/day resulting in a mean dose of 136, 67, and 17 mg XM46/kg/day for the high-, mid-, and low-dose groups, respectively. Female rats consumed approximately 18, 22, and 25 mL/day during the premating and postweaning periods resulting in a mean dose of 140, 80, and 20 mg XM46/kg/day for the high-, mid-, and low-dose groups, respectively. During gestation the dose increased to 230, 120, and 30, and during lactation the dose was 375, 220, and 50 mg XM46/kg/day for each group, respectively. Because of the wide variation in water consumption of the female rats, a median value was used to determine the overall dose of 155, 107, and 25 mg XM46/kg/day for the respective dose groups.

No clinical signs of motor skills loss were noted during the study. This was confirmed by the Opto-Varimex tests during the course of the study, where no differences in locomotor skills were found in treated or control animals.

One half of the male rats (six/group) were necropsied following the mating period (28 days treatment). Treatment-related splenomegaly (splenic enlargement) was evident. Relative spleen weights were increased by 400%, 290%, and 140% at the high-, mid-, and low-dose, respectively. Following 90 days of treatment, the remaining male rats were sacrificed. Relative spleen weights measured at sacrifice were increased by 550%, 290%, and 130% at the high-, mid-, and low-dose levels, respectively. No differences were noted at either sacrifice period in absolute or relative weights of liver, kidneys, testes, epididymides, brain, or thymus.

For male rats sacrificed following mating and those sacrificed following 90-days of treatment, sperm concentration, motility, and morphology of treated rats did not differ significantly from that of the controls.

Splenomegaly was also evident in the female rats sacrificed after 90 days of treatment. Relative spleen weights were increased by 645%, 375%, and 128% in the high-, mid-, and low-dose groups, respectively. Relative kidney weights of the high-dose female rats were also significantly increased over control values. No treatment-related differences were noted in absolute or relative weights of liver, brain, or thymus.

A treatment-related decrease in red blood cells (RBC), hemoglobin (HGB), and mean corpuscular hemoglobin concentration (MCHC) occurred in both sexes of rats at the conclusion of the study. These same parameters also showed a significant decrease in male rats following 28 days of treatment. Also, a treatment-related increase occurred in mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values. After 28 days of treatment there were no abnormalities noted in the selected clinical chemistry parameters measured though there were differences between treated and control male and female rats following 90 days of treatment.

Mean phosphorus, magnesium, total bilirubin, and uric acid values were significantly increased in both male and female high-dose groups. A decrease was noted in glucose in both sexes, while decreases in sodium and cholesterol values were noted in the female high-dose group only.

Methemoglobin concentrations measured at the conclusion of the study were higher in both male and female rats (Table 6.3-1). Among males, levels were increased by approximately 475%, 425%, and 200% over control values in the high-, mid-, and low-dose treatment groups, respectively. One half of the female rats (six/group) received untreated (no XM46) water for 24 h prior to sacrifice, while the

remaining rats continued on treated water until sacrifice. Female rats receiving water that did not contain XM46 for 24 h had methemoglobin values approximately 275%, 280%, and 125% above control values, while those receiving XM46-treated water until sacrifice were increased approximately 580%, 450%, and 175% over controls.

TABLE 6.3-1. METHEMOGLOBIN VALUES (%)[a] OF RATS FOLLOWING 90 DAYS OF TREATMENT WITH XM46

	XM46 Dose			
	Control	Low	Medium	High
Males	1.13 ± < 0.1	2.22 ± < 0.01[b]	4.93 ± < 0.1[b]	5.38 ± 0.1[b]
Females	1.13 ± < 0.1	2.00 ± < 0.1[b]	5.23 ± < 0.1[b]	6.60 ± 0.5[b]
Females[c]	1.27 ± < 0.1	1.70 ± < 0.1[b]	3.55 ± 0.1[b]	3.51 ± 0.2[b]

[a] Mean ± SEM, N = 6.
[b] Significantly different from control at $p < 0.01$.
[c] Animals on untreated water 12 h prior to sacrifice.

At necropsy all rats utilized in this study were in good general condition. Splenomegaly was consistently observed in mid- and high-dose groups of both sexes and sporadically in the low-dose male group. Discoloration/pigmentation of the kidneys ranging from dark brown to purple mottling was frequently observed in all groups, including controls. Gross examination of the pups revealed lesions in only two animals, both from control dams.

Histopathology

Observed histopathologic lesions of statistical, clinical, and pathologic significance were limited to the liver, spleen, kidney, and bone marrow. Hemosiderosis was observed in livers at increased incidence and/or severity in the mid- and high-dose female rats after 90 days of treatment. Hepatic extramedullary hematopoiesis (EMH) was increased in the mid- and high-dose groups of both sexes. This was also observed in male rats sacrificed after 28 days of treatment with XM46.

Hemosiderosis of the spleen was increased in all groups of treated females (in severity, not incidence) and in the mid- and high-dose male groups. The incidence of splenomegaly was significantly increased in the mid- and high-dose groups of both sexes. Renal tubular pigmentation was increased in the mid- and high-dose female and high-dose male groups after 90 days of treatment. Hypercellular bone marrow (myeloid hyperplasia) also showed a significant increase in incidence in the mid- and high-dose groups of both sexes. Splenomegaly and bone marrow myeloid hyperplasia were observed in the male rats sacrificed after 28 days of treatment.

Reproductive Indices

The treatment showed no adverse effects on mating because 100% of the animals mated (Table 6.3-2). The fertility index was 90% in groups given the control and high-dose treatment, but was 100% in the mid- and low-dose groups. No significant treatment-related differences were noted in length of gestation, sex ratio, gestation index, or mean number of offspring per litter. During the 21-day lactation phase, mean pup weights showed no statistically significant difference between treated and control groups.

TABLE 6.3-2. LITTER DATA FOR RATS TREATED WITH XM46

	XM46 Dose			
	Control	**Low**	**Medium**	**High**
No. of Mated Pairs	12	12	12	12
No. of Copulated Pairs	12	12	12	12
No. of Dams with Pups born	11	12	12	11
No. of Dams with Pups alive	11	12	12	11
Gestation Index (%)[a]	91.7	100.0	100.0	91.7
Live Birth Index (%)[b]	100.0	97.6	96.4	99.3
4-Day Survival Index (%)	98.8	95.7	98.1	95.1
7-Day Survival Index (%)	100.0	100.0	100.0	98.8
14-Day Survival Index (%)	100.0	98.9	100.0	100.0
21-Day Survival Index (%)	100.0	100.0	100.0	100.0
Lactation Index (%)[c]	100.0	98.9	100.0	98.8

[a] $\frac{\text{Number of females with live litters}}{\text{Number of females pregnant}}$

[b] $\frac{\text{Number of live pups at birth}}{\text{Total number of pups born}}$

[c] $\frac{\text{Number of pups surviving 21 days}}{\text{Number of pups surviving 4 days}}$

DISCUSSION

Administering XM46 in the drinking water of male and female Sprague-Dawley rats produced no adverse effects on reproductive performance, litter, or pup parameters. The clinical pathology and pathologic findings in the treated rats are characteristic of nitrate poisoning (Valli, 1993) and are similar to findings in rabbits following dermal exposure to XM46 (Asaki, 1982). Significant lesions and

abnormalities noted in the clinical pathology data are attributed to the effects of XM46 on erythrocytes. Exposure to high doses of nitrates can result in hemolytic anemia and icterus (Valli, 1993), though icterus was not observed in this study. Nitrates that are converted to high levels of nitrites can produce methemoglobinemia (Menzer, 1991).

Gross examination failed to reveal the brown discoloration of blood characteristic of methemoglobinemia (Valli, 1993); however, chemical analysis did reveal its presence. Icterus was not evident at necropsy and although total bilirubin tended to increase in a dose-related manner, the values were within normal reference ranges. Anemia was noted, using hemoglobin concentration and red blood cell count decreases as the basis of comparison. Females showed statistically significant dose-related decreases in hematocrit (HCT), but the values were within the normal reference range. Therefore, based on RBC and hemoglobin values, what was observed was a compensated hemolytic anemia (Duncan and Prasse, 1987).

It is hypothesized that at the time blood and tissues were collected for examination the bone marrow had compensated for the hemolytic anemia. The hemolysis is interpreted as occurring extravascularly based on the gross and histologic evidence of hypersplenism and the decreased MCHC. The increased MCH and MCV and decreased MCHC indicate the presence of large numbers of circulating reticulocytes (Duncan and Prasse, 1987). Because of their larger size and the exposure period allowing for a continued maximal bone marrow response, marked reticulocytosis could elevate the hematocrit into the low normal range. Unfortunately, automated hematology analyzers do not count reticulocytes or nucleated red blood cells, and blood smears were not taken from these animals.

The increased white blood cell count (leukocytosis; primarily in the females) is attributed to an absolute neutrophilia, which is common in hemolytic conditions and may be in response to erythrocyte breakdown products or stress. The eosinopenia observed in the females is most likely induced by stress. The monocytosis observed in the males may also be due to stress, but frequently accompanies the need for phagocytic removal of abnormal red cells seen in hemolytic anemia (Duncan and Prasse, 1987). Increases in total serum bilirubin is indicative of the hemolytic breakdown. An Increase in mean phosphorus and magnesium levels is also related to lyses of erythrocytes. The cause of the increase in uric acid levels is unknown. An increase in glucose levels is of questionable relevance in rats (Meeks, 1989; Loeb, 1989). Slight increases in the total bilirubin concentration with increasing dose is consistent with an extravascular hemolytic anemia (Duncan and Prasse, 1987). The consistent, and at times, statistically significant dose-related elevations of magnesium and phosphorus may be the result of hemolysis, although the ion in the highest concentrations in RBCs (potassium) is only slightly elevated (Riley and Cornelius, 1989). This may be an adaptive response because potassium concentration is regulated by a sensitive homeostatic mechanism.

Gross and histological examination supports the clinical pathologic interpretations of a compensated extravascular hemolytic anemia. Two key anatomical features for this diagnosis are splenomegaly, due to EMH and/or increased phagocytosis of damaged erythrocytes by splenic macrophages with resulting hemosiderosis, and a hypercellular erythroid bone marrow. These lesions are present in this study and follow a dose-response relationship that is statistically significant. Related hepatic lesions include hemosiderosis and EMH (erythroid). Another related and statistically significant change is pigmentation (hemosiderosis) of renal tubular epithelial cells.

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REFERENCES

Asaki, A.E., L.W. Metker, J.G. Harvey, and E.A. Haight. 1982. Health hazard evaluation of liquid monopropellants, Phase 3, Range findings studies on the effects of hydroxylammonium nitrate in animals, Special Study No. 75-51-0132-84. U.S. Army Environmental Hygiene Agency, Aberdeen Proving Ground, MD.

Bakke, J. 1990. Evaluation of the potential of LP1846 liquid gun propellant to induce unscheduled DNA synthesis in the *In Vitro* hepatocyte DNA repair assay. Final Report, SRI Project LSC-7662-2, SRI Study No. 7662-A01-89. U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD.

Balter, N., R. Davis, G. Cortes, M. Rosario, and B. Rivera. 1982. Determination of the acute oral LD_{50} and dominant lethal effects of HAN in mice. Omni Research, Inc., Baltimore, MD, Contract No. 82-MD-441, U.S. Army Environmental Hygiene Agency, Aberdeen Proving Ground, MD.

Blachman, D. 1990. An assessment of the clastogenic potential of LP1846 utilizing the mammalian cell cytogenetics assay with Chinese hamster ovary (CHO) cells. Final Report, SRI Project No. LSC-7662-4, SRI Study No. 7662-C01-89, Project No. 89M4505. U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD.

Duncan, J.R. and K.W. Prasse. 1987. *Veterinary Laboratory Medicine: Clinical Pathology.* Iowa State University Press, Ames, Iowa. pp. 1–27, 236–238.

Gross, P. 1985. Biological activity of hydroxylamine: A Review. *CRC Crit. Rev. Toxicol.* **14**(1):87–115.

Grosselin, R.E, R.P. Smith, and H.C. Hodge. 1984. *Clinical Toxicology of Commercial Products.* 5th Edition.

Hansen, R., E. Backoff, H.J. DeGrieff, and N. Klein. 1990. Processes for assessing the thermal stability of HAN-based liquid propellants, Final Report. U.S. Army Ballistics Research Laboratory Contractor Report 635. U.S. Army Ballistics Research Laboratory, Aberdeen Proving Ground, MD 21005.

Jagannath, D.R. 1979. Mutagenic evaluation of HAN 13.2 Batch #R149/151 in the Ames *Salmonella*/Microsome plate test. Final Report, Litton Bionetics, Inc., Kensington, MD. LBI Project No. 20988, U.S. Army Environmental Hygiene Agency, Aberdeen Proving Ground, MD.

Jet Propulsion Laboratory. 1989. Safe liquid propellant technology. Report on liquid propellant logistics systems study. Phase II. JPL Report D-6876. U.S. Army Armament Research, Development and Engineering Center.

Justus, J.D. and D.W. Korte. 1988. Ocular irritation evaluation with eye wash regimen of liquid propellant 1846. Institute Report No. 299, Letterman Army Institute of Research, Presidio of San Francisco, CA 94129.

Klein, N., C.S. Leveritt, and P.G. Baer. 1991. The effects of composition variation and nitric acid on the stability and reactivity of the HAN-based liquid propellants. U.S. Army Ballistics Research laboratory Technical Report 3179. U.S. Army Ballistics Research Laboratory, Aberdeen Proving Ground, MD 21005.

Loeb, W.F. 1989. The primate. In: *The Clinical Chemistry of Laboratory Animals.* W.F. Loeb and F.W. Quimby, eds. Pergamon Press, New York, New York. pp. 56–60.

Meeks, R.G. 1989. The rat. In: *The Clinical Chemistry of Laboratory Animals.* W.F. Loeb and F.W. Quimby, eds. Pergamon Press, New York, New York. pp. 19–25.

Menzer, R.E. 1991. Water and soil pollutants. In: *Casarett and Doull's Toxicology: The Basic Science of Poisons*, Fourth Edition. M.O. Amdur, J. Doull, and C.D. Klaassen, eds. Pergamon Press, New York, New York. pp. 893–894.

Parmer, D.L., W.G. Palmer, D.A. Smart, and R.A. Finch. 1991. Developmental medical research on liquid gun propellants. In: *Conference on Chemical Risk Assessment in the DOD: Science, Policy, and Practice.* H.J. Clewell III ed., ACGIH, Cincinnati, pp. 95–104.

Riley, J.H. and L.M. Cornelius. 1989. Electrolytes, blood gases, and acid base balance. In: *The Clinical Chemistry of Laboratory Animals.* W.F. Loeb and F.W. Quimby, eds. Pergamon Press, New York, New York. pp. 360–372.

Rudd, C.J. and P.S. Lee. 1990. CHO/HGPRT gene mutation assay of LP1846 liquid gun propellant. Final Report, SRI Project No. LSC-7662-3, SRI Study No. 7662-B01-89. U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD.

Snodgrass, H.L., A.E. Asaki, J.G. Harvey, and L.W. Metker. 1985. Health hazard evaluation of liquid monopropellants, Phase 4, Subchronic inhalation of hydroxylammonium nitrate. Special Study No. 75-51-1-0132-85, U.S. Army Environmental Hygiene Agency, Aberdeen Proving Ground, MD.

Valli, V.E.O. 1993. The hematopoietic system. In: *Pathology of Domestic Animals*, Vol. 3. K.V.F. Jubb, P.C. Kennedy, and N. Palmer, eds. Academic Press, New York, New York. pp. 208–209.

Weller, R.E., J.E. Morris, B.J. McClanahan, and R.F. Jostes. 1989. Evaluate the dermal toxicity of nitrate compounds and liquid gun propellants. Final Report, Project Number 87PP7806, U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD.

Witt, [illegible]M., R.M. Parker, J.D. Thurman, and P.A. Gosnell. 1992. LP1846 Liquid Gun Propellant Dermal Toxic[illegible] Study in Male Miniature Hanford Swine. Final Report, Project No. 3E162720A835, U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD.

6.4 REPRODUCTIVE TOXICITY SCREEN OF AMMONIUM DINITRAMIDE ADMINISTERED IN THE DRINKING WATER OF SPRAGUE-DAWLEY RATS

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ABSTRACT

The Department of Defense is currently considering replacing ammonium perchlorate (AP) with ammonium dinitramide (ADN). The ADN, a class 1.1 explosive oxidizer, will be used in solid rocket propellant mixtures and explosives. This study was intended to evaluate the potential of ADN to produce alterations in paternal fertility, maternal pregnancy and lactation, and growth and development of offspring. Male and female rats received drinking water treated with 200, 100, or 20 mg ADN/100 mL throughout the study. Mating occurred following 14 days of treatment. All dams, one-half the males, and representative pups were maintained for a total of 90 days of treatment. No mortality occurred in parental animals during the study. Treatment with ADN resulted in no adverse effects on mating as 92–100% of the animals mated. No treatment-related effects were seen in parental animals clinically or histopathologically. Adverse treatment-related decreases were noted in the maternal and paternal fertility indices, gestational indices, and live birth indices of the high- and mid-dose groups. Litter sizes in the high- and mid-dose groups were significantly smaller than those of the low-dose and control groups. Mean pup weights showed no statistically significant differences between ADN-treated pups and controls. Gross and histopathological examination of the animals failed to identify the cause for the decrease in litter production in the high- and mid-dose dams. This study indicates that ADN is a reproductive toxicant. The no observable effect level (NOEL) is 29 mg/kg/day, the median dose of the low-level female rats.

INTRODUCTION

The Department of Defense is currently considering replacing ammonium perchlorate (AP) with ammonium dinitramide (ADN), a class 1.1 explosive oxidizer, for use in solid rocket engine propellent mixtures and explosives. The immediate application for ADN is as a clean-burning replacement for AP in a propellent formulation that provides better performance. Burning AP produces a heavy smoke trail with high hydrochloric acid content. Chlorine in the smoke trail has been identified as a major contributor to

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ozone depletion in the stratosphere, and the visible smoke trail makes rockets more vulnerable to detection and tracking. The dinitramide anion is a uniquely stable, high oxygen density grouping that can be prepared in many different combinations including the ammonium salt that should be of most interest for rocket propulsion. Since the dinitramide anion has never been investigated before, there is great interest in its properties.

A minimal amount of acute toxicity data on ADN are available. Ammonium dinitramide did not kill rabbits at the dermal EPA exposure limit test dose of 2 g/kg body weight. The oral median lethal dose (LD_{50}) of ADN in male Fischer 344 rats is 832 mg/kg (Kinkead and Wolfe, 1994). Mortality was observed within an hour of dosing and death was preceded by convulsions. No repeated exposure toxicity information is currently available; however, field reports from exposed personnel indicate that the compound is readily absorbed by the skin, resulting in numbness of the fingers (Koppes, 1993).

Munition workers exposed to other nitrate containing explosives (e.g., nitrotoluene and 1,3-dinitrobenzene) have experienced skin irritation, liver damage, and anemia (Hathaway, 1977; Morton et al., 1976; Stewart et al., 1945). In laboratory animals, these compounds induced methemoglobin formation, liver and spleen hypertrophy, and degeneration of the seminiferous tubules resulting in decreased spermatogenesis (Cody et al., 1981; Levine et al., 1983, 1984; Furedi et al., 1984a,b). A recently completed range-finding reproduction toxicity screen performed in this laboratory with 1,3,5-trinitrobenzene resulted in testicular atrophy and decreased spermatogenesis in male rats (Kinkead et al., 1994a). Head tilt and loss of equilibrium occurred in the high-dose female rats during the postpartum period and both sexes had brain lesions in the olivary region of the medulla at the conclusion of the range-finding study. Head tilt and loss of equilibrium (Linder et al., 1990), as well as brain and brain stem lesions (Philbert et al., 1987), have been reported in rats following treatment with similar nitrate compounds.

MATERIALS AND METHODS

Test Compound

The ammonium dinitramide [$NH_4N(NO_2)_2$] was supplied by SRI International, Menlo Park, CA. Because ADN is a DOT explosive class "A" compound, only limited quantities were stored and no archive sample was maintained in the THRU laboratory. The ADN sample was known to be contaminated with 1% to 2% ammonium nitrate (Koppes, 1993). The test compound, a water-soluble powder, was maintained in an enclosed cabinet due to light sensitivity (Koppes, 1993).

Preparation of Drinking Water Solutions

The drinking water dosing solutions were prepared every 5 to 7 days, or if consumption was greatly increased, i.e., during gestation, on an 'as needed' basis. Solutions were prepared by weighing a specific quantity of ADN and adding it to a known volume of animal drinking water (wt/vol). The animal drinking water was supplied by a commercial water conditioning system (Osmotics Incorporated, Minnetonka, MN). This system consists of an activated carbon filter, softener, and reverse osmosis filtration. The pH of the water ranged from 6.5 to 7.0.

Group Assignments and Dose Levels

	Number of Animals		Dose Level of ADN	Target Dose of ADN
Group	Males	Females	(mg/L drinking water)	(mg/kg body wt/day)[a]
Control	12	12	0	0
Low	12	12	200	16
Middle	12	12	1000	80
High	12	12	2000	160

[a] Assumed daily water consumption of 40 mL per 500 g rat.

STUDY DESIGN AND EXPERIMENTAL EVALUATIONS

The test compound was administered orally in the drinking water. The study design, experimental evaluations, and animal assessments for this study are detailed in section 6.1 of this annual report. A technical report entitled "Reproductive Toxicity Screen of Ammonium Dinitramide Administered in the Drinking Water of Sprague-Dawley Rats" by Kinkead, Wolfe, Flemming, Leahy, Caldwell, Miller, and Marit (AL/OE-TR-1994-; WRAIR-TR-1994-0015) contains details of assessment data and figures not included in this annual report.

RESULTS

Water Consumption and Calculated Dose

Water consumption decreased significantly in both sexes of treated rats when treatment began and the high-dose level consumption continued to be statistically significantly less than controls throughout the study. For the duration of the study, male rats consumed approximately 37, 45, and 44 mL/day resulting in a mean dose of 146, 88, and 17 mg ADN/kg/day for the high-, mid-, and low-dose groups, respectively. Female rats consumed approximately 24, 31, and 34 mL/day during the premating and postweaning periods resulting in a mean dose of 151, 91, and 22 mg ADN/kg/day for the high-, mid-, and low-dose groups, respectively. During gestation and lactation the dose increased to 170, 102, and 30 mg ADN/kg/day for each group, respectively. Because of the wide variation in water consumption of

the female rats, a median value was used to determine the overall dose of 162, 103, and 29 mg ADN/kg/day for the respective dose groups.

General Toxicity

No mortality occurred in parental rats during the study. No treatment-related differences were noted in mean body weights of treated male rats when compared to their respective control group. However, a treatment-related decrease in mean body weights of female rats occurred at days 14 and 20 of gestation. No clinical signs of alteration in motor skills were noted during the study. This was confirmed by the Opto-Varimex tests during the course of the study, where no differences in locomotor skills were found in treated or control animals.

One half of the male rats (6/group) were necropsied following the mating period (28 days of treatment). Following 90 days of treatment the remaining male rats were examined. No treatment-related differences in absolute or relative organ weights were noted at either sacrifice. Also, sperm concentration, motility, and morphology of treated rats did not differ significantly from that of the control rats following 28 or 90 days of treatment. Likewise, no treatment-related differences were noted in absolute or relative organ weights of the female rats necropsied after 90 days of treatment.

No treatment-related differences were noted in male rat hematologic parameters following necropsy. However, a treatment-related decrease in red blood cells (RBC) and hemoglobin (HGB) occurred in the high-dose female rats at the conclusion of the study. A treatment-related increase was observed in mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) in both the high- and mid-dose female rats. Methemoglobin concentration measured at each necropsy indicated no differences between treated and control rats.

A decrease in mean serum potassium levels was noted at 28 and 90 days in the mid- and high-dose groups of both sexes. The hypokalemia ranged from a decrease of 24% in the mid-dose male rats necropsied at 28 days to 18% in the high-dose rats necropsied at that same time period.

At necropsy, all rats utilized in this study were in good general condition. Marked dilation of the uterus by clear fluid mixed with remnants of fetal skeletons was observed in one control female rat. Nodular masses were observed in the subcutaneous tissue of a mid-dose female rat and attached to the thoracic surface of the diaphragm of a low-dose male rat. Atrophy of the seminal vesicles was observed in four male rats representing all treatment groups except controls. Other gross observations included an enlarged, yellow, mottled liver in a control male and a fluid-filled scrotum in a low-dose male. Gross examination of pups revealed a renal cyst in a pup from a low-dose dam, and one case of renal discoloration in a pup from a control dam.

Histopathology

Statistical analysis revealed a significant ($p < 0.01$) decreased incidence of nephropathy in mid- and high-dose males at 28 days and decreased incidence of renal hyaline casts in the high-dose males at 90 days. There was an increased incidence of renal tubular alteration in the high-dose female rats. Mid- and high-dose females had a significant ($p < 0.01$) decreased incidence of uterine ceroid-lipofuscinosis. There were no statistically significant differences in the average severity scores for any lesion.

Reproductive Indices

The ADN-treated drinking water had no adverse effects on mating as 92% to 100% of the animals mated (Table 6.4-1). Nine of the 11 control dams and 11 of 12 low-dose dams that mated produced litters. Only 3 of the 12 mated mid-dose dams and 1 of the 11 mated high–dose dams produced litters. Litter sizes (mean of 7 pups) in the high- and mid-dose groups were significantly ($p < 0.01$) smaller than the litter sizes of the low-dose and control groups (mean of 16 pups). The gestational index showed an adverse treatment-related effect at the high- and mid-dose levels. No significant treatment-related differences were noted in the length of gestation or in the sex ratio of the pups. During the 21-day lactation phase, mean pups weights showed no statistically significant difference between treated and control groups.

DISCUSSION

Previous reproductive screens on nitrate-containing explosive compounds performed in this laboratory produced both clinical and histopathologic signs of hemolytic anemia and methemoglobinemia (Kinkead et al., 1994b,c) as well as brain lesions and loss of motor skills (Kinkead et al., 1994a). Exposure to high concentrations of nitrates can result in hemolytic anemia (Valli, 1993). Nitrates that are converted to high levels of nitrites can produce methemoglobinemia (Mener, 1991). Administering ADN in the drinking water of male and female Sprague-Dawley rats did not produce the typical clinical pathology or pathologic findings that are characteristic of nitrate poisoning.

Significant findings noted in the hematology data can be attributed to the effects of ADN on erythrocytes. Anemia is an absolute decrease in hematocrit, hemoglobin concentration, and red blood cell count. Although there was the tendency for a dose-related decrease in these parameters following 90 days of exposure to ADN in both sexes, statistically significant differences were observed only in the high-dose females (RBC and HGB, only). Significant increases in MCV and MCH in the mid- and high-dose females could be due to dehydration (which would also tend to artificially increase MCHC and HCT values) or to reticulocytosis, a bone marrow response to anemia. Neither of these conditions can be confirmed in this study. The only significant clinical finding was a loss of serum potassium in the mid- and high-dose groups of both sexes at all time points. A common cause of potassium depletion is an

increased rate of excretion by either the kidneys or the gastrointestinal tract (Mudge, 1985). The rats in the treated groups did not display signs of diarrhea during the study, and the mid- and high-dose rats had a lower water intake during the course of the study, which would infer that their renal excretion rate was not abnormally high. Although food consumption was not measured in this study, mean body weights of the hypokalemic rat groups did not differ significantly from controls indicating that these groups probably had normal dietary intake. Mohammed et al. (1992) reported a study in which placental transfer of potassium was unaltered in fetuses during maternal hypokalemia. Fetal umbilical arterial plasma concentrations were higher than maternal plasma concentrations and comparable to fetal concentrations of control animals.

The failure of the high- and mid-dose dams to produce live litters appears to be the major effect noted in this study. Gross and histopathological examination of the animals failed to identify a cause for the lack of the fetuses in the high- and mid-dose dams. It is the absence of full term pregnancy in these groups that is most likely responsible for the decreased incidence of uterine ceroid-lipofuscinosis noted in those dams. Lipofuscin is a golden-brown, finely-granular, intracellular pigment derived from the breakdown products of lipids (usually from cell membranes). It is most commonly observed in aged or chronically injured cells; therefore, it is commonly referred to as a "wear and tear" pigment. Ceroid is a variant of lipofuscin. Distinguishing the two usually requires special stains (acid fast). In this study, the pigment was observed in the uterine muscle (myometrium) or broad ligament. Stretching of the uterus during pregnancy results in membrane damage to myocytes causing deposition of this pigment. In contrast, females that did not conceive/carry pups to term did not have this change. The pigment, in this location, is not pathologically significant. The significant differences in the incidence of renal lesions are not interpreted to be biologically significant as they are common background lesions.

The results of this study indicate that ADN is a reproductive toxicant. The NOEL is 29 mg/kg/day, the median dose of the low-dose female animals.

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TABLE 6.4-1. LITTER DATA FOR RATS TREATED WITH ADN

	ADN Dose			
	Control	**Low**	**Medium**	**High**
No. of Mated Pairs	12	12	12	12
No. of Copulated Pairs	11	12	12	11
No. of Dams with Pups Born	9	11	3	1
No. of Dams with Pups Alive	9	11	3	1
Mean No. of Pups per Litter	15.3	16.4	6.7[a]	7.0[a]
Gestation Index (%)[b]	81.8	91.7	25.0[a]	10.0[a]
Live Birth Index (%)[c]	97.9	98.9	95.2	100.0
4-Day Survival Index (%)	95.6	98.9	100.0	100.0
7-Day Survival Index (%)	100.0	100.0	82.3	100.0
14-Day Survival Index (%)	100.0	100.0	100.0	100.0
21-Day Survival Index (%)	100.0	100.0	100.0	100.0
Lactation Index (%)[d]	100.0	100.0	82.3	100.0

[a] Significantly different than control at $p < 0.01$.

[b] Number of females with live litters / Number of females pregnant

(Pregnancy determined by weight gain from gestation day 0 to gestation day 7)

[c] Number of live pups at birth / Total number of pups born

[d] Number of pups surviving 21 days / Number of pups surviving 4 days

REFERENCES

Cody, T.E., S. Witherup, L. Hastings, K. Stemmer, and R.T. Christian. 1981. 1,3-Dinitrobenzene: Toxic effect *in vivo* and *in vitro*. *J. Toxicol. Environ. Health* 7(5):829–847.

Furedi, E.M., B.S. Levine, D.E. Gordon, V.S. Rac, and P.M. Lish. 1984a. Determination of the chronic mammalian toxicologic effects of TNT (Twenty-four month chronic toxicity/carcinogenicity study of trinitrotoluene (TNT) in the Fischer 344 rat). Final Report - Phase III, Vol. 1. IIT Research Institute, Project No. L6116, Study No. 11, Chicago, IL. DAMD17-79-C-9120. AD-A168 754.

Furedi, E.M., B.S. Levine, J.W. Sagartz, V.S. Rac, and P.M. Lish. 1984b. Determination of the chronic mammalian toxicologic effects of TNT (Twenty-four month chronic toxicity/carcinogenicity study of trinitrotoluene (TNT) in the B6C3F_1 hybrid mouse). Final Report - Phase IV, Vol. 1. IIT Research Institute, Project No. L6116, Study No. 11, Chicago, IL. DAMD17-79-C-9120. AD-A168 754.

Hathaway, J.A. 1977. Trinitrotoluene: A review of reported dose-related effects providing documentation for a workplace standard. *J. Occup. Med.* 19(5):341–345.

Kinkead, E.R. and R.E. Wolfe. In Press. Acute Oral and Dermal Toxicity of Ammonium dinitramide (ADN). *Acute Toxicity Data*.

Kinkead, E.R., R.E. Wolfe, S.A. Salins, C.D. Flemming, D.J. Caldwell, C.R. Miller, and J.R. Latendresse. 1994a. Range-Finding Study for a Reproductive Assessment of 1,3,5-Trinitrobenzene Administered in the Diet of Sprague-Dawley Rats. AL-TR-1994-072/WRAIR-TR-94-0006. Wright-Patterson Air Force Base, OH: Armstrong Laboratory and Walter Reed Army Institute of Research.

Kinkead, E.R., R.E. Wolfe, C.D. Flemming, D.J. Caldwell, C.R. Miller, and G.B. Marit. In Press. Reproductive Toxicity Screen of 1,3,5-Trinitrobenzene Administered in the Diet of Sprague-Dawley Rats. AL-TR-1994/WRAIR-TR-94-0016. Wright-Patterson Air Force Base, OH: Armstrong Laboratory and Walter Reed Army Institute of Research.

Kinkead, E.R., R.E. Wolfe, S.A. Salins, C.D. Flemming, H.F. Leahy, D.J. Caldwell, C.R. Miller, and G.B. Marit. In Press. Reproductive Toxicity Screen of Liquid Propellant XM46 Administered in the Drinking Water of Sprague-Dawley Rats. AL-TR-1994/WRAIR-TR-94-0008. Wright-Patterson Air Force Base, OH: Armstrong Laboratory and Walter Reed Army Institute of Research.

Koppes, William. 1993. Personal communication.

Levine, B.S., J.H. Rust, J.M. Burns, and P.M. Lish. 1983. Determination of the chronic mammalian toxicological effects of TNT (Twenty-six week subchronic oral toxicity study of trinitrotoluene (TNT) in the beagle dog). Phase II, Final Report, IIT Research Institute, Report No. L6116, Study no. 5, Chicago, IL. DAMD 17-79-C-9120, AD-A157 082.

Levine, B.S., E.M. Furedi, D.E. Gordon, P.M. Lish, and J.J. Barkely. 1984. Subchronic toxicity of trinitrotoluene in Fischer 344 rats. *Toxicology* 32:253–265.

Linder, R.E., L.F. Strader, R.R. Barbee, G.L. Rehnberg, and S.D. Perreault. 1990. Reproductive toxicity of a single dose of 1,3-dinitrobenzene in two ages of young adult male rats. *Fundam. Appl. Tox.* 14:284–298.

Mener, R.E. 1991. Water and soil pollutants. In: *Casarett and Doull's Toxicology: The Basic Science of Poisons*, Fourth Edition. M.O. Amdur, J. Doull, and C.D. Klaassen, eds. Pergamon Press, New York, New York. pp. 893–894.

Mohammed, T., J. Steele, C.P. Sibley, and R.D. Boyd. 1992. Effects of Maternal Hypokalemia on Unidirectional Maternofetal and Net Potassium Fluxes Across the Placenta of the Anesthetized Rat. *Placenta* 13(3):231–240.

Morton, A.R., M.V. Ranadive, and J.A. Hathaway. 1976. Biological effects of trinitrotoluene form exposure below the threshold limit value. *Am. Ind. Hyg. Assoc. J.* 37(1):56–60.

Mudge, G.H. 1985. Agents effecting volume and composition of body fluids. In: *Goodman and Gilman's The Pharmacological Basis of Therapeutics.* 7th Ed. A.G. Goodman, L.S. Goodman, T.W. Rall, and F. Murad, eds. MacMillan Publishing Co., NY. pp. 866–874.

Philbert, M.A., A.J. Gray, and T.A. Connors. 1987. Preliminary investigations into the involvement of the intestinal microfluora in CNS toxicity induced by 1,3-dinitrobenzene in male F-344 rats. *Tox Letters* 38:307–314.

Stewart, A., L.T. Witts, G. Higgins. 1945. Some early effects of exposure to trinitrotoluene. *Br. J. Ind. Med.* 2:74–82. (Cited in Hathaway, 1977)

Valli, V.E.O. 1993. The hematopoietic system. In: *Pathology of Domestic Animals*, Vol. 3. K.V.F. Jubb, P.C. Kennedy, and N. Palmer, eds. Academic Press, New York, New York. pp. 208–209.

6.5 GENOTOXICITY OF AMMONIUM DINITRAMIDE

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ABSTRACT

Ammonium dinitramide (ADN) was examined for its genetic toxicology effects using a battery of short-term mutagenicity screening assays, which included Salmonella/microsome mutagenesis (Ames test), mouse lymphoma cell mutagenesis (L5178Y-TK test), and *in vivo* mouse bone marrow micronuclei assay (MN test). Results from the Ames Test indicated that ADN (at a dose of 5000 μg/plate) was a base-pair substitution mutagen, causing about twofold (without S9) or threefold (with S9) increases of revertants in TA100 as compared with the controls. Results were negative in TA1535 and the frame shift mutation sensitive strains TA98 and TA1537. At a dose of 5000 μg/mL (highest dose tested), ADN induced mutations at the TK locus of mouse lymphoma cells, causing 40–95% (without S9) or 130–220% (with S9) increases of trifluorothymidine-resistant mutants. The *in vivo* micronuclei examination revealed a dose-dependent increase of micronucleated cells in the bone marrow of both male and female mice treated with ADN in a dose range of 62.5–750 mg/kg (single doses for three consecutive days), with a maximal induction of threefold increase at the highest dose. Toxicity, determined as a decrease in the polychromatophilic erythrocyte / normochromatic erythrocyte (PCE/NCE) ratio, was observed in the same dose range. The above results demonstrate that ADN is mutagenic to both bacteria and mammalian cells and causes chromosomal damage in mouse bone marrow cells *in vivo*.

INTRODUCTION

Background and Objective

The Department of Defense is developing a new generation of explosives and propellants for potential military and space applications (Borman, 1994). Ammonium dinitrimide (ADN) was selected as a candidate for study. ADN has the formula $NH_4N(NO_2)_2$ and is considered to be of chemical interest as a new type of oxide of nitrogen. ADN can act as an explosive, and its anticipated use is as an oxidizer in solid-fuel rocket propellants. The propellant currently used in the space shuttle is ammonium perchlorate (AP). However, burning AP produces a heavy smoke trail with high hydrochloric acid content. The chlorine in the smoke trail contibutes to ozone depletion in the stratosphere, and the visible smoke trail makes rockets more vulnerable to detection and tracking.

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A minimal amount of toxicity data on ADN exists. A dermal dose of 2 g/kg ADN was not lethal to rabbits. The oral LD_{50} value in rats is 832 mg/kg (Kinkead and Wolfe, 1994). Kinkead et al., 1994, conducted a reproductive toxicity screen of ADN administered in the drinking water of rats. Adverse effects were observed in maternal and paternal fertility indices, gestational indices, and live birth indices at doses of 200 and 100 mg ADN/100 mL. Gross and histopathological examination of the animals failed to provide an explanation of the observations; additional reproductive studies are underway. In general, the toxicity of nitrates includes blood abnormalities due to methemoglobin formation and histopathologic effects in select organs, such as the spleen, liver, and testes. Nitrates that metabolize to nitroso compounds may be tumorgenic. The genotoxicity of a compound indicates its mutagenic and carcinogenic potential in mammalian systems. No investigations have examined ADN for potential genotoxicity effects; thus, the overall objective of the study was to determine the genotoxicity associated with exposure to ADN.

Proposed Genotoxicity Studies

The *Salmonella*/mammalian microsome reverse mutation system (Ames test) measures the reversion from his- (histidine dependent) to his+ (histidine independent) induced by chemicals which cause base changes or frameshift mutations in the genome of this organism. In this assay, bacteria are exposed to the test agent with and without a metabolic activation system and plated onto minimum agar medium which is deficient in histidine. After incubation, revertant colonies are counted and compared with the number of spontaneous revertants in an untreated and/or vehicle control culture. The mutagenicity of the test agents is evident by the increased number of revertants.

The mouse lymphoma cell mutagenesis (L5178Y-TK test) detects the mutations at the thymidine kinase locus and is used to test the mutagenicity of the test agent in mammalian cell cultures. In DNA replication, the thymidine monophosphate (TMP) pool size is quite small and constant under normal growth conditions. If the TMP is replaced by other lethal TMP analogues, the cell will be killed. The phosphorylation of these analogues is mediated by the enzyme thymidine kinase (TK), which normally phosphorylates thymidine to TMP. TK-deficient cells lack this enzyme activity and therefore are resistant to the cytotoxic effect of the lethal analogues. In the mouse lymphoma cell forward mutation assay, the TK-competent L5178Y (TK+/+ or TK+/–) cells are treated with the test agents. After a certain period of expression, the cells are shifted to a selective medium containing the lethal analogue trifluorothymidine (TFT). Only the mutant cells (TK–/–) can survive under the selection condition, and the mutagenicity of the test compound is evident by the increase in the number of mutants.

The *in vivo* mouse bone marrow micronucleus assay (MN test), which detects the damage of chromosome or mitotic apparatus caused by chemicals, is used to examine the chromosome-damaging effect of the test agent. PCEs in bone marrow of rodents are used in this assay. The assay is based on an increase in the frequency of micronucleated PCEs in bone marrow of the treated animals. Micronuclei are small particles consisting of acentric fragments of chromosome or entire chromosomes, which lag behind at anaphase of cell division. After telophase these fragments may not be included in the nuclei of the daughter cells and form single or multiple micronuclei in the cytoplasm. The clastogenic effect or mitotic apparatus damaging effect of the test agent will be evident by the increased frequency of micronucleated PCEs in the bone marrow.

MATERIALS AND METHODS

The methodology for the three assays was given in detail in the report "Genotoxicity Assays of Ammonium Dinitramide" (Sharma and Zhu, 1994) and briefly described as follows.

All assays were conducted in accordance with the provisions of the U.S. Environmental Protection Agency/Toxic Substances Control Act (EPA/TSCA) Good Laboratory Practice Standards as defined in the *Federal Register* (40 CFR, Part 792, 1992) and the *TSCA Test Guidelines* (40 CFR 798.5265, 40 CFR 798.5300, and 40 CFR 5395, 1992). All the procedures were performed in accordance with the standard operating procedures of ManTech Environmental for the *Salmonella*/microsome mutagenesis assay, L5178Y mouse lymphoma cell mutagenesis, and mouse bone marrow cell micronucleus test.

Salmonella/Microsome Mutagenesis (Ames Test)

A preliminary range-finding assay was performed using TA100 to determine the test doses of ADN. Four tester strains were used in the mutagenicity assay, which include TA1535, TA100, TA1537, and TA98. Ammonium dinitramide dissolved in distilled water was tested in all four tester strains at a dose range of 0.3125-5 mg/plate with and without S9 activation. Cultures were set up in triplicates, and a second independent experiment was also conducted. Appropriate positive controls were included in each test (2-aminofluorene with S9, 20 μg/plate for both TA100 and TA98; sodium azide without S9, 2 μg/plate for TA1535; 9-aminoacridine without S9, 10 μg/plate for TA1537).

Mouse Lymphoma Cell Mutagenesis (L5178Y-TK Test)

The doses of ADN in the mutagenesis study were determined by a preliminary range finding assay using cell growth as the indicator for toxicity. Range finding doses of ADN were 0 (medium only), 0.25, 0.5, 2.5, 5, 25, 50, 250, 500, 2500, and 5000 mg/mL. In the mutagenesis studies, cells (6×10^6 cells in 10 mL medium for each culture) were treated with test agents with and without S9 mixture as the activation system. For each dose group, three cultures containing 200 cells/dish in non-selective medium were set up for viability measurement, another set of three cultures with 1×10^6 cells/dish in selective medium containing TFT were used for mutant counting. The mutant frequency was calculated and adjusted based on the survival percentage. Ethyl methanesulfonate (EMS, 250 nl/mL) without S9, and 3-methylcholanthrene (3-MCA, 2.5 μg/mL) with S9, were used as the positive controls in the assays. A second experiment was repeated separately.

***In Vivo* Mouse Bone Marrow Micronucleus Assay (MN Test)**

Swiss CD-1 mice (5 males and 5 females per group), 8–10 weeks old were used in the study. ADN dissolved in distilled water was administered by gavage dosing for 3 consecutive days (62.5–750 mg/kg/dose). Cyclophosphamide (20 mg/kg/dose by i.p. injection) was used as the positive control. Twenty-four hours after the last dosing, mice were sacrificed, bone marrow cells were collected and smears were prepared. The frequency of micronucleated cells was observed in 1000 PCEs per animal. The PCE/NCE ratio was determined by counting 1000 erythrocytes, and used as an indicator of toxicity. Micronuclei are defined as round bodies in cytoplasm with a diameter of 1/20 to 1/5 of an erythrocyte.

RESULTS

***Salmonella*/Microsome Mutagenesis (Ames Test)**

Five log doses (0.0005 to 5 mg/plate) of ADN were tested in TA100 for dose selection in the definitive study. The highest dose did not show toxicity to the tester strain, and was selected as the top dose along with five twofold dilutions.

The results of TA100, TA98, TA1535, and TA1537 are summarized in Tables 1 through 4, where the data is expressed as the average revertant number per plate from the triplicates.

TABLE 6.5-1. MUTAGENICITY ASSAY OF ADN IN AMES TEST (TA100)

	Experiment 1		Experiment 2	
Treatment	S9–	S9+	S9	S9
Control	113	130	109	128
DMSO	111	119	115	128
2-AF (20 μg)	178	248	149	1168
ADN (0.3125 mg)	101	149	128	139
ADN (0.625 mg)	129	162	143	156
ADN (1.25 mg)	133	199	176	226
ADN (2.5 mg)	197	219	217	285
ADN (5 mg)	240	378	244	37

TABLE 6.5-2. MUTAGENICITY ASSAY OF ADN IN AMES TEST (TA98)

	Experiment 1		Experiment 2	
Treatment	S9–	S9+	S9–	S9+
Control	24	36	43	48
DMSO	31	49	44	47
2-AF (20 μg)	134	2197	135	1944
ADN (0.3125 mg)	27	39	38	39
ADN (0.625 mg)	24	41	41	37
ADN (1.25 mg)	28	31	40	41
ADN (2.5 mg)	28	34	45	41
ADN (5 mg)	36	43	46	50

TABLE 6.5-3. MUTAGENICITY ASSAY OF ADN IN AMES TEST (TA1535)

	Experiment 1		Experiment 2	
Treatment	**S9–**	**S9+**	**S9–**	**S9+**
Control	13	17	13	15
DMSO	13	17	14	18
Sodium (2 μg) Azide	110	—	117	—
ADN (0.3125 mg)	15	15	14	16
ADN (0.625 mg)	15	15	15	16
ADN (1.25 mg)	15	18	14	17
ADN (2.5 mg)	14	17	15	15
ADN (5 mg)	15	18	16	17

TABLE 6.5-4. MUTAGENICITY ASSAY OF ADN IN AMES TEST (TA1537)

	Experiment 1		Experiment 2	
Treatment	**S9-**	**S9+**	**S9-**	**9+**
Control	6	7	6	9
DMSO	39	9	6	—
9-Amino-acridine (10 μg)	39	—	37	7
ADN (0.3125 mg)	6	7	8	9
ADN (0.625 mg)	7	9	7	7
ADN (1.25 mg)	8	7	7	8
ADN (2.5 mg)	8	8	8	8
ADN (5 mg)	8	9	6	

ADN significantly increased the revertant number in TA100 (with and without S9) in a dose-dependent manner. The maximal induction by 5 mg/plate of ADN was approximately twofold without S9, and further increased to threefold with the addition of S9. The compound was negative in the other three tester strains.

Mouse Lymphoma Cell Mutagenesis (L5178Y-TK Test)

The results of the range finding cell growth assay for ADN (data not shown) indicated that the highest dose tested (5000 μg/mL) inhibited cell growth about 30% ($p < 0.001$), and thus was selected as the top dose in the mutagenesis study, which included 5 two-fold diluted concentrations.

Table 6.5-5 summarizes the results of the two mutagenesis assays for ADN. ADN at doses above 250 μg/mL significantly increased the mutant number in one or both experiments. The mutation induction was not dose-dependent. Without S9 activation, ADN at 5000 μg/mL increased the mutation frequency at TK locus by approximately 40–95%. The mutagenicity of ADN was further enhanced by the addition of the S9 activation system, as indicated by a 130–220% increase of the TFT resistant mutant frequency over the controls.

TABLE 6.5-5. MUTAGENICITY OF ADN IN MOUSE LYMPHOMA CELLS

Treatment	Experiment 1					Experiment 2				
	Vc[a] Mean ± SD[f]	TFT[b]* Mean ± SD	F (10^{-6})[c]	F (Induced)[d]	Relative Frequency[e]	Vc[a] Mean ± SD	TFT[b] Mean ± SD	F (10^{-6})[c]	F (Induced)[d]	Relative Frequency[e]
Medium	274 ± 20.0	70 ± 2.9	51	0	1.00	278 ± 12.3	63 ± 2.1	46	0	1.00
EMS 250 nl/mL	196 ± 8.4**	240 ± 13.2*	244	193	4.78	170 ± 4.7**	220 ± 6.6**	258	212	5.65
50	267 ± 12.4	71 ± 1.9	53	2	1.05	242 ± 10.8*	63 ± 3.4	52	6	1.14
250	275 ± 13.1	65 ± 0.8	47	-4	0.93	209 ± 6.8**	54 ± 3.7	52	6	1.13
500	298 ± 11.4	90 ±1.4	60	9	1.18	256 ± 14.5	67 ± 6.0	53	7	1.15
2500	277 ± 15.2	81 ± 2.9*	58	7	1.14	249 ± 23.2	64 ± 3.9	52	6	1.13
5000	280 ± 13.2	99 ± 0.5**	71	19	1.38	240 ± 5.9*	106 ± 7.3**	88	43	1.94
Medium +S9	313 ± 6.2	81 ± 4.5	52	0	1.00	246 ± 3.6	50 ± 6.6	41	0	1.00
3-MCA 2.5 +S9	235 ±20.6**	206 ± 1.4**	175	124	3.40	216 ± 5.0**	151 ± 172**	140	99	3.42
50 +S9	310 ± 15.3	92 ± 9.4	59	8	1.15	222 ± 15.5	49 ± 1.2	44	3	1.07
250 +S9	282 ± 9.7*	85 ± 7.4	60	9	1.17	268 ± 9.6	67 ± 5.4*	50	9	1.22
500 +S9	278 ± 10.5*	69 ± 2.2	50	-2	0.96	267 ± 4.8	56 ± 2.9	42	1	1.03
2500 +S9	298 ± 4.9*	87 ± 3.9	59	7	1.14	243 ± 9.4	69 ± 1.2*	57	16	1.40
5000 +S9	356 ± 7.4	210 ± 12.8*	118	67	2.29	193 ± 11.9**	125 ± 8.2**	130	89	3.18

[a] VC = Viable count.
[b] TFT = TFT resistant mutants.
[c] F = Mutation frequency.
[d] F (induced) = F in treatment group − F in medium control.
[e] Relative Frequency = F in treatment group/F in medium control.
[f] Mean ± SD was calculated from triplicate cultures.
*, **: Compared with controls, $p < 0.05$ and $p < 0.005$.

The size-distribution of the TFT-resistant mutants (data not shown) indicated that the two positive reference mutagens, EMS (250 nl/mL without S9) and 3-MCA (2.5 μg/mL with S9) mainly showed a single peak of larger mutant size (0.5 mm), while ADN (with and without S9) showed both a peak for smaller size mutants (0.1–0.2 mm) and a peak for larger size mutants (0.5–0.6 mm). It has been suggested that small mutants may arise from the induction of chromosomal damage, while the large mutants arise from gene mutation (Clive D. et al., 1979).

***In Vivo* Mouse Bone Marrow Micronuclei test (MN test)**

Polychromatic erythrocytes and normochromatic erythrocytes represent the immature and mature erythrocytes in the bone marrow respectively. Toxicity of a test agent is indicated by the reduction in their ratio. ADN significantly reduced the PCE/NCE ratio in both male and female mice in a dose-dependent manner ($r = -0.946$, $p < 0.005$ in males, and $r = -0.973$, $p < 0.005$ in females), as shown in Table 6, suggesting a toxic effect of ADN on bone marrow. One way analysis of variance (ANOVA) indicates that there is no significant sex-difference in the bone marrow toxicity of ADN ($F = 0.676$, $p > 0.8$).

TABLE 6.5-6. TOXICITY OF ADN ON MOUSE BONE MARROW CELL PROLIFERATION (PCE/NCE)

Treatment	Single Dose (mg/kg b.w.)	PCE/NCE Male (Mean ± SD)	PCE/NCE Female (Mean ± SD)
Control	0	1.55 ± 0.01	1.55 ± 0.40
CP	20	0.38 ± 0.07	0.50 ± 0.10
ADN	62.5	1.54 ± 0.17	1.39 ± 0.06
ADN	125	1.16 ± 0.11	1.44 ± 0.19
ADN	250	1.18 ± 0.10	1.26 ± 0.10
ADN	500	1.01 ± 0.09	1.11 ± 0.06
ADN	750	0.65 ± 0.03	0.63 ± 0.05

Data for ADN 500 mg/kg in males and ADN 750, 500, and 62.5 mg/kg in females are summarized from 4 mice, while data for other groups are summarized from 5 mice.

The results of the micronuclei induction by ADN are presented in Table 7. The background frequency of micronucleated cells was 0.34% in males and 0.38% in females (normally between 0.2-0.5%). The positive control, CP, increased the MN frequency by about 7 fold. The test agent, ADN, caused dose-dependent induction of micronuclei ($r = 0.877$, $p < 0.05$ in males, $r = 0.979$, $p < 0.0005$ in females). The maximal induction of micronuclei by ADN (750 mg/kg/dose) was

about threefold higher than the controls. ANOVA indicates that there is no significant sex-difference in MN induction by ADN ($F = 0.412$, $p = 0.536$).

TABLE 6.5-7. MICRONUCLEI INDUCTION BY ADN IN MOUSE BONE MARROW CELLS

	Single Dose	Micronucleated Cells (%)	
Treatment	(mg/kg b.w.)	Male (Mean ± SD)	Female (Mean ± SD)
Control	0	0.34 ± 0.05	0.38 ± 0.13
CP	20	2.54 ± 0.47	2.62 ± 0.44
ADN	62.5	0.42 ± 0.04	0.30 ± 0.08
ADN	125	0.80 ± 0.19	0.48 ± 0.19
ADN	250	0.70 ± 0.07	0.60 ± 0.07
ADN	500	0.94 ± 0.16	0.85 ± 0.13
ADN	750	1.02 ± 0.19	1.00 ± 0.22

Data for ADN 500 mg/kg in males and ADN 750, 500, and 62.5 mg/kg in females are summarized from 4 mice, while data for other groups are summarized from 5 mice.

CONCLUSION

The results indicated that ADN is mutagenic to TA100, causing base-pair substitution mutations in Salmonella. Also, ADN significantly increased the TFT-resistant mutant frequency in mouse lymphoma cells. The mutagenicity of ADN was further enhanced by the S9 activation. Two peaks of mutant size (small and large) were observed in ADN-induced mutagenicity. ADN significantly increased the micronucleated cell frequency in the Swiss CD-1 mice polychromatic erythrocyte system in a dose-dependent manner, suggesting its chromosome-damage effect in the *in vivo* assay.

REFERENCES

Borman, S. 1994. Advanced energetic materials emerge for military and space applications. *Chemical and Engineering News*, January 17, pp. 18–22.

Clive, D. et al. 1979. *Mutation Res.* 59:61–108.

Kinkead, E.R. and R.E. Wolfe. In Press. Acute oral and dermal toxicity of ammonium dinitramide (ADN). *Acute Toxicity Data.*

Kinkead, E.R., R.E.Wolfe, C.D. Flemming, H.F. Leahy, D.J. Caldwell, C.R. Miller, and G.B. Marit. In Press. Reproductive toxicity screen of ammonium dinitramide administered in the drinking water of Sprague-Dawley rats. Technical Report, Armstrong Laboratory, Wright-Patterson Air Force Base, OH.

Sharma, S. and S. Zhu. In Press. Genotoxicity assays of ammonium dinitramide. Technical Report, Armstrong Laboratory, Wright-Patterson Air Force Base, OH.

6.6 GENOTOXICITY OF 1,3,3-TRINITROAZETIDINE

D.E. Dodd

ABSTRACT

1,3,3-Trinitroazetidine (TNAZ) is a highly energetic, castable explosive that is being considered by the Department of Defense for military and space applications. Toxicity information is needed to evaluate this compound for potential use as an explosive replacement. A structurally related compound, 1-nitroazetidine can be converted to 1-nitrosoazetidine, a rodent carcinogen. Genetic toxicity studies with TNAZ were initiated on the possibility that it also might convert or metabolize to a nitroso derivative. The following assays, capable of detecting mutations at the gene level, were employed: a bacterial (Ames *Salmonella*) test, an *in vitro* forward mutation assay at the hypoxanthine-guanine phosphoribosyl-transferase locus in Chinese hamster ovary cells, and an *in vivo* cytogenetic (mouse bone marrow erythrocyte micronucleus) test. Confirmatory assays were conducted for the *in vitro* bacterial and mammalian cell test systems. The results of the genetic toxicity studies indicated that TNAZ was negative in the three test systems. Thus, TNAZ was negative for the gene mutations in bacterial and mammalian cells and for chromosomal mutations of mouse bone marrow erythrocytes.

INTRODUCTION

The Air Force Armament Laboratory is investigating new explosive ingredients at their High Explosive Research and Development Facility. One compound that shows promise for replacing octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), CAS No. 2691-41-0, a highly energetic, castable explosive, is 1,3,3-trinitroazetidine (TNAZ), CAS No. 97645-24-4. HMX is used predominantly as a propellant and also in maximum-performance explosives (Rosenblatt et al., 1991). TNAZ is a white granular solid, it can be synthesized with 98% to 99% purity, and is recrystallizable from alcohols. Acute toxicity studies with TNAZ on laboratory animals indicate that it is nontoxic dermally, presents no sensitization potential, causes transient eye irritation, and is moderately toxic orally. The Air Force requested information on the genotoxicity potential of this high explosive candidate. Three mutagenic tests were selected for genotoxicity screening; the Ames *Salmonella* test, the hypoxanthine-guanine phosphoribosyl-transferase (HGPRT) test in Chinese hamster ovary (CHO) cells, and the mouse micronucleus assay.

Background Toxicity Data on TNAZ

Pursuant to the request by the U.S. Army Armament Research, Development, and Engineering Center to perform toxicity studies on TNAZ, the U.S. Army Environmental Hygiene Agency initiated acute toxicity, irritancy, and sensitization tests. Results of these tests follow (Weeks, private communication). Sprague-Dawley rats were administered single oral doses of TNAZ dissolved in dimethylsulfoxide (DMSO). The approximate lethal dose (ALD) was 2222 mg/kg; the ALD for DMSO was > 3333 mg/kg. No deaths were observed when TNAZ acetone solutions were applied dermally to the occluded, shaved skin of New Zealand White (NZW) rabbits and albino-Hartley guinea pigs at dosages up to 1480 and 3333 mg/kg, respectively. Technical grade TNAZ (50% solution in acetone) did not induce a delayed contact sensitization reaction in guinea pigs following the Buehler sensitization protocol. Primary skin and eye irritancy tests (Draize method) were performed on NZW rabbits. TNAZ was not irritating to the intact skin. Neat TNAZ produced slight injury to the cornea and moderate to severe injury to the conjuctivae. These eye irritation results were resolved within two days after exposure. In summary, TNAZ was nontoxic and nonirritating dermally, produced no sensitization potential, was of moderate toxicity orally, and caused transient eye irritation.

Proposed Genotoxicity Studies

A chemical related to TNAZ is 1-nitroazetidine. 1-Nitroazetidine can be converted to 1-nitrosoazetidine, which has been extensively investigated for its carcinogenicity and has been shown to be strongly positive in the rat, mouse, and hamster (Lijinsky et al., 1984). This information leads to the concern that TNAZ also might be converted or metabolized to corresponding nitroso derivatives that are carcinogenic. There are no literature citations on TNAZ to support or refute this proposal. Because the genotoxicity of a compound may reflect its carcinogenicity potential, three common assays were selected to investigate the genotoxicity potential of TNAZ. The assays chosen were the Ames *Salmonella*/microsomal test, the CHO/HGPRT forward mutation test, and the mouse *in vivo* bone marrow micronucleus test. These assays were chosen because of their high sensitivity and reliability.

The Ames assay detects both base pair substitution point mutations and/or frameshift mutations. A high percentage of chemicals that elicit a mutagenic response in the Ames assay are potential animal and human mutagens and carcinogens (McCann and Ames, 1976). Because the *Salmonella* assay has been shown to indicate mechanisms of chemical interaction with DNA and has produced few false positives for noncarcinogens, it has been used as the cornerstone of any battery of genotoxicity testing.

The CHO/HGPRT forward mutation assay has been used to determine the mutagenicity of chemicals in mammalian germ cells. The gene for HGPRT is located on the X-chromosome. In an evaluation of Phase III of the U.S. Environmental Protection Agency's (EPA) genetic toxicology program (Li et al., 1988), the high sensitivity value of the CHO/HGPRT assay was supported. This assay detected mutagenicity of industrial and environmental chemicals from 25 chemical classes and, of the 43 proven carcinogens tested, 40 were found to be positive.

The mouse bone marrow micronucleus assay is a rapid, *in vivo* cytogenetic assay based on the observation that cells with broken chromosomes or impairments of the spindle apparatus often have disturbances in the distribution of chromatin during cell division. Micronuclei are formed from chromosomes or chromosomal fragments left behind during anaphase. In this assay, polychromatophilic erythrocytes (PCE) in the bone marrow are scored for the presence of micronuclei. During maturation from erythroblast to erythrocyte the nucleus is extruded, whereas micronuclei, if present, remain in the cytoplasm. Detection of the micronuclei in nonnucleated cells is thus facilitated and provides a useful index of clastogenicity or anaphase-lag in erythrocytes (Schmid, 1976).

MATERIALS AND METHODS

The Toxic Hazards Research Unit subcontracted the performance of the three genotoxicity assays with a Good Laboratory Practice (GLP)-certified commercial laboratory. The statement of work for the subcontract requested that the three tests be carried out according to EPA's Health Effects Testing Guidelines (40 CFR, Parts 798.5265, 798.5300, and 798.5395, 1 July 1990 edition). Confirmatory assays were conducted for the *in vitro* bacterial and mammalian cell test systems. A brief presentation of the materials and procedures employed for each assay follows. Details of the materials and methods for each test are given in reports by Paika (1994 a,b,c).

TNAZ Test Substance

The TNAZ has a structural formula of $C_3N_4H_4O_6$ and is a white granular solid with a particle size of approximately 100 microns. The crystal density is 1.84 and the onset exotherm temperature is 200 °C. The test material was supplied by J.W. Mitchell, Jr., Director, Systems Safety, Headquarters Air Force Development Test Center (AFSC), Eglin AFB, FL. Solubility tests were performed by R.B. Nolan, Chemical Research Officer, Eglin AFB, FL. Results of the solubility tests indicated TNAZ to be nonsoluble in water, slightly (10%) soluble in saline, 100% soluble in DMSO or acetone, and nonsoluble in corn oil.

***Salmonella typhimurium* Reverse Mutation Assay (Ames assay)**

The *Salmonella typhimurium* strains used in this assay were TA98, TA100, TA1535, TA1537, and TA1538. The preincubation technique was used to enhance the sensitivity of the plate incorporation assay. The TNAZ test substance was dissolved in DMSO and administered *in vitro* directly into the test system. Bacteria were exposed to the test substance both in the presence and absence of a metabolic activation system (S9 microsomal fraction of rat liver homogenate obtained from Aroclor 1254-treated rats). Positive control substances with and without metabolic activation systems for all strains and negative control substances were used. Ten concentrations (10000 to 0.1 mg/plate) were used in the dose range finding assay; six concentrations (500 to 0.01 mg/plate) were used in the reverse mutation assay.

Gene Mutation at the HGPRT Locus in Cultured CHO Cells Assay

The CHO-K1 cell line was originally derived from the ovary of a female Chinese hamster (*Cricetalus griseus*) obtained from American Type Culture Collection, Rockville, MD. The TNAZ test substance was dissolved in 0.5% DMSO and administered *in vitro* directly into the test system. The CHO cells were exposed to the test substance both in the presence and absence of a metabolic activation system (S9 microsomal fraction of rat liver homogenate obtained from Aroclor 1254-treated rats). Positive control substances with and without metabolic activation systems and negative control (including solvent control) substances were used. Ten concentrations (5.00 to 0.008 mg/mL) were used in the dose range-finding assay; seven concentrations (0.500 to 0.008 mg/mL) were used in the activated mutagenic test; and seven concentrations (0.250 to 0.004 mg/mL) were used in the non-activated mutagenic test. Duplicate cultures seeded with 5×10^5 cells/flask were used at each dose level. Post-expression, the cells were harvested and reseeded in selection medium at 2×10^5 cells per 100 mm dish. Five dishes were plated per dose level for a total of 1×10^6 cells. Concurrently, 200 cells per 100 mm dish were seeded in complete medium for the Parallel Cloning Efficiency Assay.

Mouse Bone Marrow Erythrocyte Micronucleus Test

Healthy male and female albino Swiss mice (*Mus musculus*) were obtained from Charles River Breeding Laboratories (Wilmington, MA). The animals were 7 to 12 weeks of age at the start of the study. The TNAZ test substance was suspended in corn oil and administered by intraperitoneal injection. Mice received 3 single doses, 24 h apart. In a dose range-finding study, death was observed in mice administered doses @ 100 mg/kg (body weight). Five doses were selected for the

definitive study: 40, 20, 10, 5, and 1 mg/kg. Positive (mitomycin C) and negative (corn oil) substances also were administered to naive mice. Mice were sacrificed 24 h after the last dose. Bone marrow slides were prepared from the femur. A total of 1000 PCEs was scored for the presence of micronuclei. The slides were scored blindly to reduce bias associated with the analysis.

RESULTS

***Salmonella typhimurium* Reverse Mutation Assay (Ames Assay)**

Range-Finding Assay: The range-finding assay was performed with strain TA100, negative control substance plates, with and without microsomal activation. Some toxicity was observed, as determined by a reduction in the number of spontaneous revertants, a clearing of the background lawn, and by the degree of survival of treated cultures.

The negative control substance plates gave a reference point from which to compare the data. The negative control substance values fell within two standard deviations of the historical mean value for the laboratory or reference literature.

The mean number of revertants per plate was calculated for each concentration (data not shown). A positive result was not observed for any strain since a significant increase in the number of revertant colonies over its corresponding negative control substance was not observed. Since toxicity was detected, dose levels were chosen to bracket toxic and non-toxic levels.

Reverse Mutation Assay: The positive control substance assays consisted of direct-acting mutagens and mutagens requiring metabolic biotransformation. All positive controls exhibited twice the number of colonies as the negative control substances, demonstrating that the test system was functional with known mutagens (Tables 6.6-1 and 6.6-2). The negative control substance plates, for each strain, gave a reference point to compare the test data. Their values fell within two standard deviations of the historical or literature mean values.

TABLE 6.6-1. AMES REVERSE MUTATION ASSAY WITHOUT MICROSOMAL ACTIVATION

Strain	Revertants/Plate *							
	Controls		Test Article (TNAZ) Dose Levels (μg/plate)					
	Positive Control**	Negative Control***	500	50	5	0.5	0.5	0.01
TA98								
MEAN	166.0	33.7	0.0	31.0	32.3	32.3	32.3	31.3
SD	7.9	0.6	0.0	1.0	2.1	1.5	3.2	1.2
TA100								
MEAN	329.3	135.7	1.7	129.7	127.0	128.0	127.7	133.7
SD	12.3	7.0	2.1	4.7	6.1	2.6	1.5	5.7
TA1535								
MEAN	152.0	23.0	0.0	23.0	23.0	22.7	23.0	23.0
SD	5.2	1.0	0.0	1.0	2.6	1.5	1.0	2.6
TA1537								
MEAN	105.3	12.3	0.0	10.7	12.7	9.7	12.3	11.7
SD	1.5	0.6	0.0	0.6	0.6	0.6	1.5	1.5
TA1538								
MEAN	124.7	18.0	0.0	17.3	17.3	18.3	18.3	17.0
SD	2.1	1.0	0.0	2.1	1.5	1.5	0.6	2.0

* All plates were dosed at 100 μl/plate.

** The positive control used was sodium azide for strains TA-100 and TA-1535, 2-nitrofluorene for strain TA-98, and 9-aminoacridine for strain TA-1537.

*** The negative control used in the assay was dimethylsulfoxide.

The test substance is not considered mutagenic because the number of revertant colonies associated with the test substance did not represent a two-fold increase over the number of revertant colonies associated with the corresponding negative control substance (Tables 6.6-1 and 6.6-2). The results are considered valid since the positive control substance yielded a mutagenic response, and the values for the negative control substance fell within the 95% confidence limit of the historical background.

A dose–response was not observed for the test article in the reverse mutation assay. The results of the assay were confirmed through an independent confirmatory assay (with fresh samples).

Gene Mutation at the HGPRT Locus in Cultured CHO Cells Assay

Range Finding Assay: Of the doses of TNAZ tested, the higher dose concentrations at 5.00, 2.00, 1.00, and 0.500 mg/mL were cytotoxic in the activated system (data not shown). Therefore, 0.500, 0.250, 0.125, 0.062, 0.031, 0.016, 0.008, and 0.004 mg/mL doses were utilized.

Mutagenicity Assay: Both in the presence and absence of microsomal S-9 liver enzyme, the test substance failed to induce significantly large numbers of mutant colonies (Tables 6.6-3 and 6.6-4). The effect of the test substance on the expression of mutant colonies was similar to untreated (negative) and solvent controls, whereas both positive controls exhibited an increased induction of mutant colonies.

The mutagenicity assay was repeated as a confirmatory assay. These results indicate that the test substance did not induce increased numbers of mutants and is comparable to that of the negative control substance under the conditions utilized in this test system.

TABLE 6.6-2 AMES REVERSE MUTATION ASSAY WITH MICROSOMAL ACTIVATION

	Revertants/Plate*							
Strain	Controls		Test Article (TNAZ) Dose Levels (μg/plate)					
	Positive Control**	Negative Control***	500	50	5	0.5	0.5	0.01
TA98								
MEAN	174.3	40.3	0.0	40.3	41.3	42.7	42.3	40.3
SD	2.9	0.6	0.0	1.5	2.1	1.5	2.5	1.5
TA100								
MEAN	421.3	181.7	11.7	180.0	174.0	177.3	170.7	171.7
SD	15.5	5.7	2.5	4.6	2.0	2.5	2.1	4.0
TA1535								
MEAN	187.3	25.7	0.0	26.7	25.7	26.7	26.3	26.3
SD	3.8	2.5	0.0	0.6	2.1	0.6	1.5	0.6
TA1537								
MEAN	123.3	14.3	0.0	13.7	14.3	12.7	13.7	14.0
SD	3.8	2.1	0.0	1.5	1.2	1.2	0.6	1.0
TA1538								
MEAN	130.0	19.3	0.0	19.7	19.0	17.3	20.7	21.0
SD	5.3	1.5	0.0	0.6	1.7	3.2	1.2	2.0

* All plates were dosed at 100 μl/plate.
** The positive control used was 2-aminoanthracene for all strains.
*** The negative control used in the assay was dimethylsulfoxide.

TABLE 6.6-3. HGPRT/CHO MUTATION WITH TNAZ

Test	Results with Activation			
	Mutant Scoring		Parallel Cloning Efficiency	
	Ave. Mutant Col/Dish	Ave. Surviving Colonies	Ave. Percent Plating Efficiency	Mean Mutant Frequency Per 1 × 10^6 Survivors
Test Substance (0.500 mg/mL)	0.0	29.0	14.5	0.00
Test Substance (0.250 mg/mL)	0.4	146.0	73.0	2.74
Test Substance (0.125 mg/mL)	0.6	156.0	78.0	3.87
Test Substance (0.062 mg/mL)	0.6	150.0	75.0	4.00
Test Substance (0.032 mg/mL)	1.0	159.0	79.5	6.29
Test Substance (0.016 mg/mL)	0.8	165.0	82.5	4.85
Test Substance (0.008 mg/mL)	0.6	135.0	67.5	4.44
Negative Control	0.4	162.0	81.0	2.47
Solvent Control (0.5% DMSO)	0.6	170.0	85.0	3.53
Positive Control DMN (0.3 μL/mL)	18.6	156.0	78.0	119.23

COL = Colonies
DMN = Dimethylnitrosamine
DMSO = Dimethylsulfoxide

TABLE 6.6-4. HGPRT/CHO MUTATION WITH TNAZ

Test	Results without Activation			
	Mutant Scoring		Parallel Cloning Efficiency	
	Ave. Mutant Col/Dish	Ave. Surviving Colonies	Ave. Percent Plating Efficiency	Mean Mutant Frequency per 1×10^6 survivors
Test Substance (0.250 mg/mL)	0.0	0.0	0.0	0.0
Test Substance (0.125 mg/mL)	0.0	0.0	0.0	0.0
Test Substance (0.062 mg/mL)	0.0	0.0	0.0	0.0
Test Substance (0.032 mg/mL)	0.6	124.0	62.0	4.84
Test Substance (0.016 mg.mL)	0.8	162.0	81.0	4.94
Test Substance (0.008 mg/mL)	0.4	158.0	79.0	2.53
Test Substance (0.004 mg/mL)	0.6	146.0	73.0	4.11
Negative Control	0.4	163.0	81.5	2.45
Solvent Control (0.25% DMSO)	0.4	156.0	78.0	2.56
Positive Control 4-NQ (0.03 μL/mL)	14.6	145.0	72.5	100.69

COL = Colonies
4-NQ =4-Nitroquinolin-1-oxide
DMSO = Dimethylsulfoxide

Mouse Bone Marrow Erythrocyte Micronucleus Test

Range-Finding Assay: Immediately after dosing, death due to the toxicity of the test substance was observed among all the animals dosed at 500 mg/kg. Death also was observed in 3 out of 6 animals dosed at 100 mg/kg immediately after dosing. The remaining three animals at the 100 mg/kg dose exhibited tremors. At 10 mg/kg, signs of tremors were observed for all animals. No signs of toxicity were observed at 1.0 mg/kg.

Final Assay: For the final assay, doses were 40, 20, 10, 5.0, and 1.0 mg/kg. At the 40, 20, and 10 mg/kg dose levels, tremors were observed in all animals immediately after injection. At 5.0 mg/kg, 5 out of 10 animals exhibited tremors. The remaining 5 animals did not exhibit any signs of toxicity. No signs of toxicity were observed in any animals at the 1.0 mg/kg dose level.

There was a statistically significant increase in the number of micronucleated PCEs in the positive control substance group compared to the negative control substance group (Table 6.6-5). In the negative control substance, the average number of micronucleated polychromatic erythrocytes per 1000 PCEs did not exceed five (Table 6.6-5).

TABLE 6.6-5. MOUSE MICRONUCLEUS ASSAY WITH TNAZ

Analysis of Micronucleated Cells in Bone Marrow Extract Smears

Test	Number of Animals	Number of Micronucleated Cells/1000 Polychromatic Erythrocytes (MEAN ± SD)
Positive Control Substance*	10	40.60 ± 4.58
Negative Control Substance**	10	3.60 ± 0.70
TNAZ - 40 mg/kg	10	4.00 ± 1.05
TNAZ - 20 mg/kg	10	4.40 ± 0.97
TNAZ - 10 mg/kg	10	4.50 ± 0.85
TNAZ - 5 mg/kg	10	4.30 ± 0.82
TNAZ - 1 mg/kg	10	4.60 ± 0.84

* Mitomycin C (0.2 mg/kg in corn oil)
** Corn oil (40 mL/kg)

Each test and control group was analyzed separately for male versus female animals utilizing a Student t-test to analyze for possible sex differences. Since no statistical significance was noted in the frequency of micronuclei between males and females, the data were pooled and males and females were analyzed as a combined data set (Table 6.6-5).

The frequency of micronucleated PCEs in each dose group was compared to that of the respective negative control substance using analysis of variance and Newman-Keuls Test for confirmation of pairwise comparisons. All results are considered not significant at $p^{3}0.05$. There was a statistically significant increase in the number of micronucleated PCEs in the positive control substance group compared to the negative control substance group, at p£0.05.

The test substance did not produce a statistically significant dose-related increase in the number of micronucleated PCEs or a statistically significant and reproducible positive response at any one of the test substance concentrations.

CONCLUSION

The results of the genetic toxicity studies indicated that TNAZ was negative in all three test systems. Results of the *in vitro* bacterial and mammalian cell gene mutation assays were confirmed with independent confirmatory tests. In the three test systems, the positive control substance gave clearly positive results of mutagenicity indicating that the test systems were able to detect changes in mutation rates. Thus, TNAZ is not considered to have mutagenic potential based on the experimental conditions described and the results obtained from these three genotoxicity assays.

REFERENCES

Li, A.P., R.S. Gupta, R.H. Heflich, and J.S. Wassom. 1988. A review and analysis of the Chinese hamster ovary/hypoxanthine guanine phosphoribosyl transferase assay to determine the mutagenicity of chemical agents. A Report of Phase III of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat. Res.* 196: 17–36.

Lijinsky, W., R.M. Kovatch, and G.L. Knutsen. 1984. Carcinogenesis by nitrosomorpholines, nitrosooxazolidines and nitrosoazetidine given by gavage to Syrian golden hamsters. *Carcinogenesis* 5: 875–878.

McCann, J. and B.N. Ames. 1976. Detection of carcinogens as mutagens in the *Salmonella*/microsome test. Assay of 300 chemicals: Discussion. *Proc. Natl. Acad. Sci. U.S.A.* 72: 5135–5139.

Paika, I.J. 1994a. Genetic toxicity evaluation of 1,3,3-trinitroazetidine. Volume I of IV: Results of *Salmonella typhimurium* reverse mutation assay (Ames assay). Technical Report, in press. Armstrong Laboratory, Wright-Patterson Air Force Base, OH.

Paika, I.J. 1994b. Genetic toxicity evaluation of 1,3,3-trinitroazetidine. Volume II of IV: Results of mouse bone marrow micronucleus test. Technical Report, in press. Armstrong Laboratory, Wright-Patterson Air Force Base, OH.

Paika, I.J. 1994c. Genetic toxicity evaluation of 1,3,3-trinitroazetidine. Volume III of IV: Results of gene mutation at the HGPRT locus in cultured Chinese hamster ovary cells. Technical Report, in press. Armstrong Laboratory, Wright-Patterson Air Force Base, OH.

Rosenblatt, D.H., E.P. Burrows, W.R. Mitchell, and D.L. Palmer. 1991. Organic explosives and related compounds. In: O. Hutzinger, ed. *The Handbook of Environmental Chemistry*, Vol. 3, Part G, pp. 195–234. Berlin Heidelberg: Springer-Verlog.

Schmid, W. 1976. The micronucleus test for cytogenetic analysis. In: A. Hollaender, ed. *Chemical Mutagens* Vol. 4A, pp. 31–53. NY: Plenum Press.

Weeks, M.H. 1989. Letter to Commander, USAEHA, regarding toxicity study results with TNAZ, 21 September.

6.7 CARDIOVASCULAR SCREENING OF NITRATED EXPLOSIVES AND PROPELLANTS IN MALE SPRAGUE-DAWLEY RATS

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ABSTRACT

The effects of nitrated explosives and propellants like nitroglycerin and propylene glycol dinitrate on the cardiovascular system of munitions workers are well-documented. Symptoms of exposure vary from headaches and angina to sudden death. It is important to screen various nitrated compounds and blends to determine their immediate and long-term effects on the cardiovascular system and identify biomarkers for use in the field. The purpose of this study was to screen nitrated explosives and propellants for their long-term effects on the cardiovascular system of conscious, male Sprague-Dawley rats. Parameters evaluated were heart rate, mean blood pressure, systolic pressure, and diastolic pressure. Validation studies comparing non-invasive tailcuff sphygmomanometry and implanted telemetric devices suggest that additional testing and data evaluation be performed before final conclusions and recommendations are made regarding the continued use or abandonment of either of these methodologies. Therefore, until further notice, both procedures should be used to acquire parallel data involving toxicological effects of nitrated explosives and propellants on blood pressure and heart rate in rats.

INTRODUCTION

Nitrated explosives and propellants used by the military are heterogenous with respect to their physical, chemical and toxicological properties. Some, like nitroguanidine (Hiatt et al., 1986, 1988; Morgan et al., 1986; Brown et al., 1988; Reddy and Korte, 1988; Ho et al., 1988) and nitrocellulose (Montgomery, 1982), are relatively nontoxic. Others, like nitroglycerin and propylene glycol dinitrate, have profound pharmacologic effects (NIOSH, 1978; Carmichael and Lieben, 1963; Stewart et al., 1974; Kylin et al., 1964). Tolerance to these chemicals can lead to rebound effects when individuals are no longer exposed to them. Effects can range from mild vasoconstriction to sudden death.

Traditional methods for monitoring the effects of pharmacologically active chemicals on the cardiovascular system in animals involve direct cannulation of major arteries and direct injection of drugs into the vessels of anesthesized animals. The direct injection of drugs into anesthetized animals is not an ideal model for determining the effects of inhalation, ingestion, or dermal exposure

of conscious individuals to pharmacologically active agents. Direct cannulation requires invasive procedures for placement of cannulas, maintenance of patency in the externalized portion, and a physical connection between the exteriorized portion and a blood pressure/heart rate monitor. It has been reported that long-term, chronic in-dwelling arterial catheters affect food consumption and weight gain in rats (O'Neill and Kaufman, 1990).

A major non-invasive alternative to direct cannulation in animals is tailcuff syphygmomanometry. A variety of studies have been done to validate this procedure against direct cannulation (Lucas, 1971; Andrews and Jones, 1978; Palbol and Henningsen, 1979; Yamakoshi et al., 1979; Borg and Viberg, 1980; Bunag and Butterfield, 1982; Borkowski and Quinn, 1983; Bunag, 1983; Wen et al., 1988; Ferrari et al., 1990; Kuwahara et al., 1991; O'Neill and Kaufman, 1990; Ikeda et al., 1991; Spanos et al., 1991). A disadvantage to this method is the need to restrain the animal to minimize movements that would interefere with readings. Therefore, animals must either be trained or anesthetized. In rodents, additional problems arise because of their ability to use their tail arteries as thermoregulators. Body temperatures must be elevated to induce a tail pulse. However, excessive heating can cause artifactual increases in blood pressure (Wen et al., 1988; Kuwahara et al., 1991). Under ideal conditions, tailcuff syphygmomanometry appears to correlate well with direct cannulation although readings are generally 5 mm of mercury lower than those obtained from direct cannulation.

A third and more recent approach for measuring blood pressure is cannulation of a major artery with an implantable transmitter sewn into place inside a body cavity (Brockway et al., 1991). The technique has been validated against long-term, direct, tethered cannulation (Guiol et al., 1992; DePasquale et al., 1994). The animal is allowed to recover and then placed in its cage near a receiving unit that detects radio signals emitted by the implanted telemetry device. The receiver converts the radio waves into electronic signals that are sent to a computer and recorded as blood pressure, heart rate, temperature, electocardiograms, or combinations thereof. The principal advantage of this technique over conventional cannulation is the elimination of an exteriorized cannula that may have problems with patency and sterility. The elimination of the requirement that an animal be tethered to a blood pressure monitor removes a potential source of stress and concommittent undesirable rises in baseline blood pressure.

A developmental study at Wright-Patterson Air Force Base compared tailcuff syphygmomanometry and radiotelemetry implants in the same rats to determine if one or both of these procedures would be appropriate to use as a means for monitoring cardiovasular responses to pharmacologically active agents. Results have shown the tailcuff data to be about 20 mm lower than telemetric implant data. Preliminary analyses of tailcuff data have shown internal correlations of systolic pressure, mean blood pressure, diastolic pressure, and heart rate to be considerably

lower than the correlations acquired for implants. Weight gain in implanted animals appears to be poorer than in their nonimplanted counterparts. Whether this reduction in weight gain is due to poor eating habits or physiological stress needs to be evaluated. Therefore, the preliminary data and analyses obtained thus far require that further investigations be made prior to abandonment of either of these methodologies.

EXPERIMENTAL DESIGN

Validation Study

A. Male Sprague-Dawley rats were subjected for 5 consecutive days to 2 daily series of triplicate tailcuff analyses within an ambient temperature chamber (29–30 °C). The animals were trained in restraints so readings could be obtained without anesthesia.

B. After baseline studies were completed, some of the animals were subjected to surgery for implantation of telemetric devices into the lower abdominal aorta. The animals were allowed to recover from surgery prior to continued analyses.

C. All animals were subjected to an additional 5-day series of tailcuff readings as previously described.

D. Blood pressure (systole, diastole, and mean blood pressure) and heart rate were evaluated beginning in the morning Monday through Friday. As many as 6 valid readings per rat were taken each day by tailcuff, and 10 second scans were taken every 5 minutes throughout the day by radiotelemetry.

E. The data were consolidated into spreadsheets for statistical evaluations to compare cardiovascular parameters as measured by tailcuff sphygmomanometry before and after telemetric implantation and to compare tailcuff and telemetric implant readings obtained simultaneously from the same animals.

Long Term Studies

A. Male Sprague-Dawley rats will be divided into 4 groups (maximum of 3 per group): control, low, medium, and high dose corresponding to dose levels used in the ancillary nitrate reproductive studies.

B. The test compound will be administered either by diet or drinking water. Animals to be tested will be from the same lot used in the ancillary reproductive study.

C. Experimental Evaluations

1. Body Weight: Body weights will be determined in conjunction with animals in the reproductive study and will be used to determine dosages of test compound consumed.

2. Food or Water Consumption: Food or water consumption will be determined in conjunction with animals in the reproductive study and will be used to determine dosages of test compound consumed.

3. Cardiovascular Monitoring: Blood pressure (systole, diastole, and mean blood pressure) and heart rate will be evaluated beginning in the morning Monday through Friday. As many as three valid readings per rat will be taken each day and averaged.

Statistical Analysis

Statistical procedures will be based on the assumption of a normal distribution. A one-factorial, multivariate analysis of variance with multiple comparisons will be done using Bonferroni adjustment. It will be assumed that variance is equal among dose groups and each group is normally distributed. We will test for equality of variance using the multivariate Leven's test for normality using the Wilk-Shapiro test. If one of the above assumptions is found not to be homogenous, appropriate transformations of the data will be used, e.g., log, rank, etc.

RESULTS

Blood pressure and heart rate data from 5 male Sprague-Dawley rats were acquired 1 week prior to implantation (base cuff) and one week post implantation (exp cuff and implants). Descriptive statistics for systolic, mean blood pressure, diastolic, and heart rate data are described in Tables 6.7-1 through 6.7-4. There were no significant differences noted between tailcuff readings taken before and after implantation as determined by a two-sample, multivariant, independent t-test analysis. However, except for systolic readings, all postimplantation tailcuff readings were slightly higher than preimplantation readings. The tailcuff blood pressure and heart rate readings were significantly lower than those obtained by telemetry ($p < 0.01$). The magnitude of the differences was considerably greater between blood pressure readings than between heart rate readings. Tailcuff readings for systole, mean blood pressure, diastole, and heart rate prior to implantation were 119, 90, 75, and 339, respectively. Postimplantation tailcuff readings were 118, 94, 81, and 341, and telemetric readings were 136, 114, 97, and 364.

Pearson correlation coefficients were obtained from within each group of data, i.e., base cuff, exp cuff, and implants (Table 6.7-5). Comparisons were made between systole versus mean blood pressure (SYS/MBP), systole versus diastole (SYS/DIA), mean blood pressure versus diastole (MBP/DIA), systole versus heart rate (SYS/HR), mean blood pressure versus heart rate (MBP/HR),

and diastole versus heart rate (DIA/HR). Significant correlations at $p < 0.0001$ in all three data groups were detected when systolic, mean blood pressure, and diastolic readings were compared to each other. Correlations were considerably lower in tailcuff data as compared to implant data with the exception of the MBP/DIA group. Correlations ranged from 0.40 to 0.50 and 0.65 to 0.72 for tailcuff comparisons between systole and diastole and systole and mean blood pressure, respectively. Tailcuff correlations for mean blood pressure and diastole were much higher (0.96). Correlations for implant comparisons between systole and mean blood pressure, systole and diastole, and mean blood pressure and diastole were 0.98, 0.93, and 0.97, respectively. Correlations of blood pressure parameters (systole, mean blood pressure, and diastole) to heart rate in tailcuff data were poor and/or inversely related. Similar correlations in implant data were higher, positively related, and highly significant ($p < 0.0001$), but still relatively low. Tailcuff correlations of systole to heart rate, mean blood pressure to heart rate, and diastole to heart rate ranged from −0.43 to −0.13, −0.21 to 0.10, and −0.09 to 0.18, respectively. Telemetric correlations of systole to heart rate, mean blood pressure to heart rate, and diastole to heart rate were 0.37, 0.44, and 0.45, respectively.

Tables 6.7-1 through 6.7-4 includes descriptive statistics on 5 male Sprague-Dawley rats 1 week prior to implantation (base cuff) and 1 week post implantation (exp cuff and implants). Tables 6.7-1 through, 6.7-4 represent descriptive statistics for systole, mean blood pressure, diastole, and heart rate, respectively. Readings in columns 2 and 3 were acquired by tailcuff sphygmomanometry. Those in column 4 were acquired by radiotelemetry. Readings in columns 3 and 4 were acquired on the same days. Readings were taken during 5 consecutive days before implantation and 5 consecutive days after implantation. The statistics described in column 1 include: total number of readings for all 5 animals (N), average of all readings (MEAN), standard deviation from the mean (SD), standard error of the mean (SEM), coefficient of variation (CV), minimum reading (MIN), maximum reading (MAX), and range of readings (RANGE).

TABLE 6.7-1. SYSTOLE

	Base Cuff	Exp Cuff	Implants
N	113	108	392
MEAN	119	118	136*
SD	16		
SEM	1.51	1.49	0.53
CV	0.14	0.13	0.08
MIN	83	76	112
MAX	165	151	185
RANGE	82	74	72

TABLE 6.7-2. MEAN BLOOD PRESSURE

	Base Cuff	Exp Cuff	Implants
N	113	108	392
MEAN	90	94	114*
SD	16.9	16.5	8.9
SEM	1.59	1.59	0.45
CV	0.19	0.18	0.08
MIN	51	55	94
MAX	129	143	157
RANGE	78	89	63

* = significantly different than exp cuff at $p < 0.01$.

TABLE 6.7-3. DIASTOLE

	Base Cuff	Exp Cuff	Implants
N	113	108	392
MEAN	75	81	97*
SD	21.0	19.9	7.7
SEM	1.98	1.92	0.39
CV	0.28	0.24	0.08
MIN	14	40	79
MAX	113	140	131
RANGE	99	100	52

* = significantly different than exp cuff at $p < 0.01$.

TABLE 6.7-4. HEART RATE

	Base Cuff	Exp Cuff	Implants
N	113	108	392
MEAN	339	341	364*
SD	34.5	31.0	43.3
SEM	3.24	2.98	2.19
CV	0.10	0.09	0.12
MIN	267	269	281
MAX	492	432	490
RANGE	224	163	209

* = significantly different than exp cuff at $p < 0.01$.

Table 6.7-5 includes Pearson correlation coefficients on 5 male Sprague-Dawley rats 1 week prior to implantation (base cuff) and 1 week post implantation (exp cuff and implants). Readings in columns 2 and 3 were acquired by tailcuff sphygmomanometry. Those in column 4 were acquired by radiotelemetry. Readings in columns 3 and 4 were acquired on the same days. Readings were taken during 5 consecutive days before implantation and 5 consecutive days after implantation. The correlations described in column 1 are comparisons within each group and include: systole versus mean blood pressure (SYS/MBP), systole versus diastole (SYS/DIA), mean blood pressure versus diastole (MBP/DIA), systole versus heart rate (SYS/HR), mean blood pressure versus heart rate (MBP/HR), and diastole versus heart rate (DIA/HR).

TABLE 6.7-5. Correlation Analyses

	Base Cuff	Exp Cuff	Implants
SYS/MBP	0.65**	0.72**	0.98**
SYS/DIA	0.40**	0.50**	0.93**
MBP/DIA	0.96**	0.96**	0.97**
SYS/HR	−0.43**	−0.13	0.37**
MBP/HR	−0.21*	0.10	0.44**
DIA/HR	−0.09	0.18	0.45**

*$p < 0.03$, **$p < 0.0001$.

DISCUSSION

At the present time, it appears that the radiotelemetric implant procedure has several advantages over tailcuff sphygmomanometry: (1) It is far more convenient to monitor the animals because they require no restraints, and hence no training. (2) Internal correlations of systole, mean blood pressure, and diastole are considerably higher than they are in the tailcuff procedure. (3) Readings can be taken immediately with the implant method whereas with the tailcuff procedure, the animals have to be heated for at least thirty minutes up to 30 °C to get adequate readings. Even with this procedure, some of the animals fail to generate adequate pulses. Implantation did not appear to have an effect on future blood pressure readings in older animals as determined by comparing differences in tailcuff readings between pre-implanted and post-implanted animals.

Disadvantages of the radiotelemetric procedure include the requirement for invasive surgery and an apparent significant impact on animal weight gain. Two animals implanted at a weight of about 300 grams had an average weight of 401 grams compared to an average weight of 504 grams for 15 animals not yet implanted, though the latter were about 1 week younger than the former. This is a serious problem, since lack of weight gain indicates physiological stress within the animal. External correlation studies suggest that modest correlations (0.21–0.27) exist between tailcuff and telemetric implant diastolic, mean blood pressure, and heart rate data, but not between systolic pressures.

Preliminary experiments suggest that preheating the animals to higher temperatures prior to placing them into the ambient chamber may help correct the poor readings obtained in some animals. It remains to be seen if it will also help reduce the time needed to obtain pulses and/or improve internal correlation data. Without direct arterial cannulation studies, it is impossible to say whether the tailcuff or the implant data are more accurate in terms of absolute blood pressure. The

tailcuff data are in good agreement with some other studies described in the literature (Bidani et al., 1993; Bunag and Butterfield, 1982), including one study involving radiotelemetric implants (Guiol et al., 1992). However, higher blood pressure ranges for tailcuff studies in Sprague-Dawley rats have also been described (Spanos et al., 1991).

Since these data are important in determining which procedure (tailcuff, implants, or both) will be used to monitor changes in blood pressure and/or heart rate in response to exposure of animals to nitrated explosives and/or propellants, absolute blood pressure and heart rate measurements may be less critical than the ability of the procedure(s) to detect changes in data from baseline values in response to exposure to nitrated compounds.

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REFERENCES

Andrews, D.I. and D.R. Jones. 1978. Comparison of a tail-cuff method of measuring rat blood pressure with direct arterial recordings of blood pressure in conscious rats. *Proc. Univ. Otago Med. Sch.* 56:3–4

Bidani, A.K., K.A. Griffin, M. Picken, and D.M. Lansky. 1993. Continuous telemetric blood pressure monitoring and glomerular injury in the rat remnant kidney model. *Amer. Physiol. Soc.* F391–F398.

Borg, E. and A. Viberg. 1980. Role of heating in non-invasive blood pressure measurements in rats. *Acta Physiol. Scand.* 108:73–75.

Borkowski K.R. and P. Quinn. 1983. Validation of indirect systolic blood pressure measurement in ether anaesthetised rats. *J. Auton. Pharmac.* 3:157–160.

Brockway, B.P., P.A. Mills, and S.H. Azar. 1991. A new method for continuous chronic measurement and recording of blood pressure, heart rate and activity in the rat via radio-telemetry. *Clin. Exper. Hyper.-Theory and Practice.* A13:885–895.

Brown, L.D., C.R. Wheeler, and D.W. Korte. 1988. Oral toxicity of nitroguanidine in male and female rats. Letterman Army Institute of Research, LAIR Report No. 264. Presidio of San Francisco, CA.

Bunag, R. D. 1983. Facts and fallacies about measuring blood pressure in rats. *Clin. Exper. Hyper.-Theory and Practice.* A5:1659–1681.

Bunag, R.D. and J. Butterfield. 1982. Tail-cuff blood pressure measurement without external preheating in awake rats. *Hypertension.* 4:898–903.

Carmichael, P. and J. Lieben. 1963. Sudden death in explosive workers. *Archives of Environmental Health*. 7:50–65.

Depasquale, M.J., L.W. Ringer, R.L. Winslow, R.A. Buchholz, and A.A. Fossa. 1994. Chronic monitoring of cardiovascular function in the conscious guinea pig using radio-telemetry. *Clin. Exper. Hypertension.* 16:245–260.

Ferrari, A.U., A. Daffonchio, F. Albergati, P. Bertoli, and G. Mancia. 1990. Intra-arterial pressure alterations during tail-cuff blood pressure measurements in normotensive and hypertensive rats. *J. Hypertension.* 8:909–911.

Guiol, C., C. Ledoussal, and J.M. Surge. 1992. A radiotelemetry system for chronic measurement of blood pressure and heart rate in the unrestrained rat validation of the method. *J. Pharmacol Toxicol. Methods.* 28:99–105.

Hiatt, G. F. S., S.K. Sano, and D.W. Korte. 1986. Primary eye irritation potential of nitroguanidine in rabbits. Letterman Army Institute of Research, AD-A164675. Presidio of San Francisco, San Francisco, CA.

Hiatt, G.F.S., S.K. Sano, C.R. Wheeler, and D.W. Korte. 1988. Acute oral toxicity of nitroguanidine in mice. Letterman Army Institute of Research, LAIR report No. 265. Presidio of San Francisco, San Francisco, CA.

Ho, B., J.A. Tillotson, L.S. Kincannon, P.B. Simboli, and D.W. Korte, Jr. 1988. The fate of nitroguanidine in the rat. Fund. Appl. *Toxicol.* 10:438–458.

Ikeda, K., Y. Nara, and Y. Yamori. 1991. Indirect systolic and mean blood pressure determination by a new tailcuff method in spontaneously hypertensive rats. *Lab. Animals.* 25:26–29.

Kuwahara, M., S. Sugano, K.I. Yayou, H. Tsubone, and H. Kobayashi. 1991. Evaluation of a new tail-cuff method for blood pressure measurement in rats with special reference to the effects of ambient temperature. *Exp. Anim.* 40:331–336.

Kylin, B.A., H. Englund, S Ehiner. and S. Yllner. 1964. A comparative study on the toxicology of nitroglycerin, nitroglycol and propylene glycol dinitrate. *International Congress of Occup. Health.* Proc. 15th. Vienna. 3: 191–195.

Lucas, J.A. 1971. A modified indirect method of blood pressure measurement in the conscious and anaesthetized rat. *Proc. Physiol. Soc.* 218:1P–3P.

Montgomery, R. 1982. Polymers. *Patty's Industrial Hygiene and Toxicology*. 3rd Ed., lic. 4385–4386.

Morgan, E.W., S.K. Sano, and D.W. Korte. 1986. Primary dermal irritation potential of nitroguanidine in rabbits. Letterman Army Institute of Research, LAIR Report No. 220. Presidio of San Francisco, San Francisco, CA.

NIOSH. 1978. Criteria for a recommended standard. *Occupational exposure to nitroglycerin and ethylene glycol dinitrate.*

O'Neill, P.J. and L.N. Kaufman. 1990. Effects of indwelling arterial catheters or physical restraint on food consumption and growth patterns of rats: advantages of noninvasive blood pressure measurement techniques. *Lab. Animal Sci.* 40:641–643.

Palbol, J. and J. Henningsen. 1979. Blood pressure measurements in the conscious rat. *Scand. J. Urol. Nephrol.* 13:319–321.

Reddy, G. and D.W. Korte. 1988. Mammalian toxicity studies with nitroguanidine. *Proceedings of the 13th Annual Environmental Quality R&D Symposium.* 436–449.

Ruben, D.B. and J. Butterfield. 1982. Tail-cuff blood pressure measurement without external preheating in awake rats. *Hypertension.* 4:898–903.

Spanos, H.G., R. DiNicolantionio, and T.O. Morgan. 1991. The use of non-invasive blood pressure measurements to measure pressor responses in rats during air stress. *Clin. Exp. Pharmacol. Physiol.* 18:761–765.

Stewart R.D., J.E. Peterson, P.E. Newton, C.L. Hake, M.J. Hosko, A.J. Lebrun, and G.M. Lawton. 1974. Experimental human exposure to propylene glycol dinitrate. *Toxicol. Appl. Pharmacol.* 30:377–395.

Wen, S.F, J.M. Trembley, M. Qu, and J.G. Webster. 1988. An impedence method for blood pressure measurement in awake rats without preheating. *Hypertension.* 11:371–375.

Yamakoshi, K.I., H. Shimazu, and T. Togawa. 1979. Indirect measurement of instantaneous arterial blood pressure in the rat. *Amer. J. Physiol.* 237:H632-H637.

7.1 THE TOXICOLOGIC AND ONCOGENIC POTENTIAL OF JP-4 VAPOR: 90-DAY CONTINUOUS INHALATION EXPOSURE

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ABSTRACT

Male and female Fischer-344 rats, female C57BL/6 mice, and male and female beagle dogs were divided into three treatment groups and exposed nearly continuously (23 h/day, 7 days/week) to JP-4 jet fuel vapor at concentrations of 0, 500, and 1000 mg/m^3 for 90 days. At exposure termination, all dogs and 1/3 of the rodents were euthanatized and the remaining animals were held for either a 19- or 21-month postexposure tumorigenesis observation period. Pathologic findings in male rats revealed treatment-related renal toxicity consistent with male rat $\alpha_{2\mu}$-globulin nephropathy. No treatment-related respiratory toxicity was noted in any species. This study did not demonstrate target-organ toxicity or carcinogenesis which could be extrapolated to other species.

INTRODUCTION

JP-4 is a complex mixture of aliphatic and aromatic hydrocarbon compounds with physicochemical properties similar to gasoline. It was the principal fuel used in U.S. Air Force flight operations until conversion to JP-8 and meets Military Specification MIL-J-5624E. When this study was initiated (1979), JP-4 fuel accounted for approximately 85% of the turbine engine fuel used by the Department of Defense. Because of its widespread use within the military, investigations were conducted to determine the acute, subchronic, and chronic toxicity of JP-4 vapor.

Single oral doses of neat fuel were administered to Sprague-Dawley rats at 8 g JP-4/kg. No deaths were observed. In CF_1 mice, deaths occurred at lower doses (JP-4 diluted in corn oil) of 0.5 g/kg, but only 33% mortality was achieved at the highest gavage dose of 1.0 g JP-4/kg. An inhalation study of 6 h duration to an estimated JP-4 concentration of 38,000 mg/m^3 (based on weight loss) resulted in poor coordination and convulsions in several rats, but no mortality (Kinkead et al., 1993). Dermal sensitization studies with guinea pigs indicated that JP-4 was not a skin sensitizer. Rabbit eye irritation tests were negative while dermal application of the jet fuel resulted in slight skin irritation in that same species (Kinkead et al., 1992). An 8-month inhalation study (6 h/day, 5 days/week) at 5000 mg JP-4/m^3 to beagle dogs, Rhesus monkeys, Sprague-Dawley rats, and C57BL/6 mice produced increased organ-to-body weight ratios of liver, kidney, lung, and splenic tissue, bronchial irritation in male rats, and a

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transient increase in osmotic erythrocyte fragility in female dogs. Histopathologic findings in exposed monkeys, dogs, and mice showed no treatment-related effects (MacEwen and Vernot, 1975).

In a subsequent study, male and female Fischer-344 (F-344) rats and male and female C57BL/6 mice were exposed to 1000 and 5000 mg JP-4/m^3, 6 h/day, 5 days/week, for 12 months. The inhalation exposure period was followed by a 12-month holding/observation period for possible oncogenic effects (Bruner et al., 1993). Pathologic findings in male rats revealed treatment-related renal toxicity and neoplasia consistent with male rat $\alpha_{2\mu}$-globulin nephropathy syndrome. The study did not produce any additional target-organ toxicity or tumor formation that was considered treatment related.

Results of inhalation exposure to fuels similar in composition to JP-4 have been reported. Kinkead et al. (1991) reported the effects of a one-year (6 h/day, 5 days/week) study with the fuels JP-TS, a high altitude jet fuel, and JP-7, a fuel similar to JP-5 jet fuel, on F-344 rats and C57BL/6 mice. The most significant finding was an increased incidence of renal disease in male rats similar to changes reported with other hydrocarbon inhalation exposures (Carpenter et al., 1975a,b). Continuous exposure (24 h/day, 7 days/week) of F-344 rats, C57BL/6 mice, and beagle dogs to 750 and 150 mg JP-5/m^3 vapor for 90 days also resulted in similar renal changes in male rats (Gaworski et al., 1984). A 90-day continuous exposure (24 h/day, 7 days/week) of F-344 rats and C57BL/6 mice to 500 and 1000 mg/m^3 JP-8 vapor only resulted in the renal changes in male rats (Mattie et al., 1991).

This study was designed to determine the toxicologic effects of a 90-day nearly continuous (approximately 23 h/day) exposure of dogs, rats, and mice to atmospheric concentrations of 500 and 1000 mg JP-4/m^3. This exposure regimen simulates worst-case working conditions that may be experienced by military personnel (Mattie et al., 1991). Beagle dogs, F-344 rats, and C57BL/6 mice were selected as the test species to afford a comparison with the above-mentioned studies. Rodents were held for an additional 19- to 21-month postexposure to assess the oncogenic potential of JP-4.

MATERIALS AND METHODS

Details on the test material characteristics, generation and analysis methods, analytical findings, and general study design are detailed in the 1980 Toxic Hazards Research Unit Annual Technical Report (MacEwen and Vernot, 1980).

RESULTS

Clinical Findings and Mortality

Clinical signs of toxic stress were not apparent during or following the 90-day inhalation exposure regimen. No treatment-related differences in mortality occurred during the exposure period. Mean body weights of both sexes were significantly depressed at many of the weighing periods throughout the 90-day study (data not shown). The mean body weights of the JP-4-exposed female rats recovered during the postexposure observation period, while the depression in mean body weight continued through much of the postexposure period in the JP-4-exposed male rats. Mouse mean weights revealed no exposure-related effects. The mean body weights of dogs exposed to JP-4 vapor continuously for 90 days were unaffected by exposure.

Hematology and Clinical Chemistry

Mean hematology and blood chemistry values for male and female rats following the 90-day exposure showed no treatment-related effects. Likewise, no treatment-related differences in these parameters were noted in dogs at any of the sampling times. All clinical measurements made on dog blood samples were within normal species variation and no treatment-related changes in osmotic fragility were noted (data not shown). Blood chemistry evaluations performed on male and female rats postexposure also showed no treatment-related effects (data not shown).

Organ Weights

A treatment-related increase in absolute and relative kidney weights was observed in the male rat groups euthanatized following the 90-day exposure period (Table 7.1-1). Relative spleen weights were also slightly increased in the treated male rat groups. Additionally, absolute liver weights were decreased in the low concentration male rats. This difference was not a factor when corrected for the difference in whole body weight. No significant concentration-related differences in mean absolute or relative organ weights were observed in the female rat groups. No treatment-related differences were noted in dog organ weights. No differences occurred in organ weights of either male or female rats measured following the postexposure period.

TABLE 7.1-1. ORGAN WEIGHTS (g)[a] AND ORGAN TO BODY WEIGHT RATIOS[b] OF F-344 RATS FOLLOWING 90-DAY CONTINUOUS EXPOSURE TO JP-4 VAPOR

	Control	500 mg/m^3	1000 mg/m^3
Males			
Liver	8.30 ± 0.13	7.57 ± 0.14[c]	8.08 ± 0.18
Ratio	2.56 ± 0.02	2.47 ± 0.04	2.61 ± 0.04
Spleen	0.55 ± 0.01	0.57 ± 0.01	0.58 ± 0.02
Ratio	0.17 ± < 0.01	0.19 ± < 0.01[c]	0.19 ± < 0.01[c]
Kidneys	2.04 ± 0.03	2.11 ± 0.04	2.31 ± 0.04[c]
Ratio	0.63 ± 0.01	0.69 ± 0.01[c]	0.75 ± 0.01[c]
Whole Body	324.3 ± 3.6	306.0 ± 3.3[c]	309.6 ± 4.2[c]
Females			
Liver	4.30 ± 0.11	4.10 ± 0.08	4.24 ± 0.05
Ratio	2.50 ± 0.04	2.46 ± 0.02	2.57 ± 0.03
Spleen	0.37 ± 0.01	0.34 ± 0.01	0.37 ± 0.02
Ratio	0.21 ± < 0.01	0.21 ± < 0.01	0.22 ± < 0.01
Kidneys	1.24 ± 0.03	1.22 ± 0.03	1.24 ± 0.02
Ratio	0.72 ± 0.01	0.73 ± 0.01	0.75 ± 0.01
Whole Body	172.1 ± 2.6	166.8 ± 1.8	164.8 ± 1.5[d]

[a] Mean ± SEM, Males N = 25; Females N = 24.
[b] Organ weight/body weight × 100.
[c] Significantly different from control, $p < 0.01$.
[d] Significantly different from control, $p < 0.05$.

Dog Pathology

Gross examination of dogs, all euthanatized immediately postexposure, revealed roundworm infection in 10 of 12 test dogs and 1 of 6 control dogs. All microscopic lesions observed in dogs were regarded as spontaneous changes or natural diseases in laboratory beagles. In all groups the most significant finding was mild to moderate inflammatory changes in the lungs and associated lymphoid tissues. These changes were not treatment related, and in almost all cases, entirely compatible with infection by canine lungworms, *Filaroides sp.*

Rat Pathology

Histopathologic findings in rats following the 90-day inhalation exposure were confined to the kidneys of male rats. Hyaline droplet formation was present in 100% of the 1000 mg JP-4/m^3 group and 96% of the 500 mg JP-4/m^3 group, as opposed to only 7% of the control group (Table 7.1-2). This lesion consisted of the formation of hyaline crystalloid intracytoplasmic inclusions. Additionally, granular casts were present in 100% of the high-concentration group and 96% of the low-concentration group. Casts were not observed in any of the rats from the control group. A slight increase in severity of these two lesions was present in the high-concentration group.

TABLE 7.1-2. INCIDENCE SUMMARY OF SELECTED MICROSCOPIC RENAL LESIONS IN F-344 RATS EXAMINED IMMEDIATELY FOLLOWING 90 DAYS OF CONTINUOUS EXPOSURE TO JP-4 VAPOR

	Male			Female		
	Control	500 mg/m^3	1000 mg/m^3	Control	500 mg/m^3	1000 mg/m^3
Number Examined	27	27	27	25	25	25
Nephropathy (severity)[a]	0 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)	1 (0.0)	0 (0.0)
Tubular Mineralization (severity)	0 (0.0)	1 (0.0)	1 (0.0)	0 (0.0)	3 (0.1)	0 (0.0)
Granular Protein Casts (severity)	0 (0.0)	25[b] (1.6)	27[b] (2.3)	0 (0.0)	0 (0.0)	1 (0.1)
Hyaline Droplet Accumulation (severity)	2 (0.1)	26[b] (1.0)[b]	27[b] (1.9)[b]	0 (0.0)	0 (0.0)	1 (0.1)
Pelvic Urothelium Hyperplasia (severity)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a] Mean severity score based on 0 = normal; 1 = minimal; 2 = mild; 3 = moderate; and 4 = severe. Score is the mean score of the affected animals.
[b] Significantly different from control, $p < 0.01$.

Mouse Pathology

The most significant finding in mice following the 90-day inhalation exposure was hepatocellular vacuolization. This lesion was found in a high percentage of the low-concentration group (89%) and the high-concentration group (90%) as opposed to control mice (4%). This finding was most prominent in the centrolobular region of the liver. The lesion consisted of multiple, discrete vacuoles of varying sizes within the cytoplasm of hepatocytes.

Acute inflammatory changes consisting of infiltrates of eosinophils in the submucosa of the gallbladder were seen with slightly greater frequency in the JP-4-exposed mice (22% and 33%, high- and low-exposed group, respectively) when compared with controls (9%). Frequently, but not exclusively, this change was accompanied by hyaline degeneration changes of the mucosal epithelium.

Renal tubular dilation was noted with slightly greater frequency in the low-concentration mice (29%) when compared with the controls (7%) and high-concentration (10%) mice. This lesion consisted of slightly dilated tubules near the corticomedullary junction which were filled with pink, homogeneous fluid.

Ultrastructural Examination

Examination of kidney ultrastructure of JP-4-exposed male rats revealed hyaline, crystalloid intracytoplasmic inclusions in the proximal tubule cells. The severity of the inclusions was greater in the high dose group, although occasional proximal tubule cells from the low-concentration group contained crystalloid inclusions equal in size and number to the high-concentration proximal tubules. Mitochondria and endoplasmic reticulum, which were not affected until proximal tubules became engorged with inclusions, exhibited excessive dilation of the albuminal cell membrane, or began to form a cast. The appearance of the mitochondria then became more irregular in shape and the cristae appeared more dilated than in the control group. The casts were composed of necrotic, exfoliated tubular epithelial cells which formed prominent tubular plugs near the corticomedullary junction and resulted in focal tubular dilation. Distal tubules appeared normal in both low and high dose groups. Glomeruli were unaffected by JP-4 exposure.

Histopathology — Postexposure Results

Histopathologic findings at 19- and 21-month postexposure were limited to male rats. Protein casts and hyaline droplets were no longer significant findings in the kidneys of treated animals (Tables 7.1-3 and 7.1-4). Instead, an increased incidence of medullary tubular mineralization and hyperplasia of pelvic urothelium was noted.

No JP-4-related lesions were noted in the mice examined postexposure. Incidence of lesions noted in the JP-4-exposed mouse groups did not differ significantly from those of the control mouse group.

TABLE 7.1-3. INCIDENCE SUMMARY OF SELECTED MICROSCOPIC RENAL LESIONS IN F-344 RATS 19 MONTHS AFTER A 90-DAY CONTINUOUS EXPOSURE TO JP-4 VAPOR

	Male			Female		
	Control	500 mg/m^3	1000 mg/m^3	Control	500 mg/m^3	1000 mg/m^3
Number Examined	31	26	27	31	25	22
Nephropathy	29	24	25	7	11	11
(severity)[a]	(1.7)	(1.8)	(2.2)	(0.2)	(0.5)	(0.5)
Tubular Mineralization	2	25[b]	26[b]	1	0	1
(severity)	(0.1)	(1.9)[b]	(2.1)[b]	(0.0)	(0.0)	(0.0)
Granular Protein Casts	0	0	1	6	0	2
(severity)	(0.0)	(0.0)	(0.0)	(0.2)	(0.0)	(0.1)
Hyaline Droplet Accumulation	0	0	0	1	0	0
(severity)	(0.0)	(0.0)	(0.0)	(0.1)	(0.0)	(0.0)
Pelvic Urothelium Hyperplasia	1	7	16[b]	0	0	0
(severity)	(0.0)	(0.3)	(1.1)[b]	(0.0)	(0.0)	(0.0)

[a]Mean severity score based on 0 = normal; 1 = minimal; 2 = mild; 3 = moderate; and 4 = severe. Score is the mean score of the affected animals.
[b]Significantly different from control, $p < 0.01$.

TABLE 7.1-4. INCIDENCE SUMMARY OF SELECTED MICROSCOPIC RENAL LESIONS IN F-344 RATS 21 MONTHS AFTER A 90-DAY CONTINUOUS EXPOSURE TO JP-4 VAPOR

	Male			Female		
	Control	500 mg/m^3	1000 mg/m^3	Control	500 mg/m^3	1000 mg/m^3
Number Examined	16	21	20	15	20	18
Nephropathy	16	21	20	8	17	10
(severity)[a]	(1.9)	(2.5)[b]	(2.5)[b]	(0.7)	(1.0)	(0.7)
Tubular Mineralization	0	21[c]	19[c]	0	1	0
(severity)	(0.0)	(1.7)[c]	(2.0)[c]	(0.0)	(0.1)	(0.0)
Granular Protein Casts	0	0	0	2	1	5
(severity)	(0.0)	(0.0)	(0.0)	(0.1)	(0.1)	(0.3)
Hyaline Droplet Accumulation	0	0	0	0	0	0
(severity)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)
Pelvic Urothelium Hyperplasia	1	17[c]	17[c]	0	0	0
(severity)	(0.1)	(2.0)[c]	(1.9)[c]	(0.0)	(0.0)	(0.0)

[a]Mean severity score based on 0 = normal; 1 = minimal; 2 = mild; 3 = moderate; and 4 = severe. Score is the mean score of the affected animals.
[b]Significantly different from control, $p < 0.05$.
[c]Significantly different from control, $p < 0.01$.

DISCUSSION

Pathophysiological changes noted in dogs, mice, and rats following 90 days of continuous exposure to JP-4 vapor indicated no significant respiratory toxicity. Except for a depression in mean body weight gains of rats, all species tolerated the exposures without adverse clinical signs or increase in morbidity or mortality. Hematologic and clinical chemistry evaluations conducted on rats immediately following exposure, and again at 19 months postexposure, did not identify changes considered to be of toxicological significance.

Significant microscopic lesions following the 90-day continuous exposure were primarily restricted to renal lesions found in male rats. The renal lesions were consistent with those characterized in previous studies (Kinkead et al., 1991; Carpenter et al., 1975a,b; Gaworski et al., 1984; Mattie et al., 1991). The hyaline droplets formed in proximal tubular epithelial cells have been shown to consist of $\alpha_{2\mu}$-globulin (Swenberg et al., 1989). This protein is synthesized in extraordinary amounts in the liver of male rats exposed to hydrocarbons and is under the control of androgens. Alpha$_{2\mu}$-globulin is filtered by the glomerulus and resorbed by the epithelial cells of the proximal convoluted tubules forming the hyaline droplets. The hyaline crystalloid inclusions, found in the ultrastructural examination of the kidneys, are now known to represent this protein within phagolysosomes (Mattie et al., 1991). The granular protein casts observed in this and other studies represent entrapped cellular debris most likely from the proximal convoluted tubular epithelium, although concurrent necrosis was not observed. Ultrastructurally, these casts were located near the junctions of the pars recta and the descending limb of the loop of Henle.

By 19 months, postexposure hyaline droplets and protein casts had resolved and the principal lesions were linear concretions of the inner medulla thought to be mineralized necrotic debris trapped in the hairpin turn of the loops of Henle and, in the high-exposure group, hyperplasia of the renal pelvic urothelium. Hyperplasia of the pelvic urothelium is often associated with mineralization that extends into the renal pelvis and may be due to mineral-induced rigidity of the renal papilla which results in friction/stimulation of the overlying pelvic urothelium. Each of these changes were also the lesions of significance noted at the final necropsy (21-month postexposure). Chronic progressive nephrosis was a background lesion affecting control and exposed animals of both sexes. Although the incidence of this lesion was similar for all groups, the severity was greatest for those males exposed to JP-4 vapor, suggesting some interaction between $\alpha_{2\mu}$-globulin nephropathy and chronic, progressive nephrosis.

Although several neoplasms were encountered in rats, there were no statistically significant differences in incidence between treated and control groups. The numbers and types of tumors were within the expected historical normal range for aging F-344 rats (Haseman et al., 1990). Only one renal tumor was noted in this study, a male control rat examined 21-month postexposure. Bruner et al. (1993) observed that renal tumor incidence was increased in male rats that had been exposed for at least one year to hydrocarbons which induce $\alpha_{2\mu}$-globulin nephropathy. Renal tumors were not observed in similar

hydrocarbon studies in which the animals were held up to 21-month postexposure following 90 days of continuous exposure. Bruner et al. (1993) concluded that increased renal neoplasia may be expected when male rats were exposed for one year or longer to hydrocarbons which promote tubular cell death (and proliferation) following perturbations in the renal handling of $\alpha_{2\mu}$-globulin .

The hepatocellular vacuolization noted in mice examined at exposure termination was presumably an indication of fatty change. The presence of this lesion was undoubtedly exposure related, occurring in a large majority of exposed mice. Reversibility was demonstrated by the reduced incidence of fatty changes in exposed mice examined postexposure. With no other signs of liver damage noted in exposed mice, the fatty changes can be regarded as a mild, reversible response to JP-4 vapor exposure.

Renal tubular dilation found in mice following the 90-day exposure is produced by a fluid which is thought to originate from an incompetent glomerular filtration mechanism secondary to mild membranous glomerulonephritis. Glomerulonephritis is a lesion common in aging C57BL/6 mice (Frith et al., 1983) and was equally distributed between treated and control mouse groups at the 19- and 21-month examination.

CONCLUSION

The most significant finding in this study was the increased incidence of renal disease in the male rats. No renal neoplasms were found in the JP-4-exposed male rats. Many organic chemicals, including military aviation fuels can produce hyaline droplet nephropathy in the male rat (Bruner, 1984; Bruner et al., 1993; Kinkead et al., 1974, 1991; Gaworski et al., 1984, 1985; Mattie et al., 1991). The mechanisms associated with this syndrome is specific for the male rats and involves the production and accumulation of $\alpha_{2\mu}$-globulin . Renal hyperplasia and neoplasia develop subsequently (Alden et al., 1984; Ridder et al., 1990; Borghaff et al., 1991). Because $\alpha_{2\mu}$-globulin is nonexistent in humans, chemicals producing nephropathy and/or renal tumors via this mechanism are not considered to produce similar renal effects in humans.

REFERENCES

Alden, C.L., R.L. Kanerva, G. Ridder, and L.C. Stone. 1984. The pathogenesis of the nephrotoxicity of volatile hydrocarbons in the male rat. In: *Advances in Modern Environmental Toxicology: Renal Effects of Petroleum Hydrocarbons.* M.A. Mehlman, C.P. Hemstreet III, J.J. Thorpe, and N.K. Weaver, eds. Vol. VII. New Jersey: Princeton Scientific Publishers, Inc.

Borghaff, S.J., A.B. Miller, J.P. Bowen, and J.A. Swenberg. 1991. Characteristics of chemical binding to $\alpha_{2\mu}$-globulin *in vitro*: Evaluating structure-activity relationships. *Toxicol. Appl. Pharmacol.* 107:228–238.

Bruner, R.H. 1984. Pathologic findings in laboratory animals exposed to hydrocarbon fuels of military interest. In: *Renal Effects of Petroleum Hydrocarbons.* M.A. Mehlman, C.P. Hemstreet, J.J. Thorpe III, and N.K. Weaver, eds. pp. 133–140. New Jersey: Princeton Scientific Publishers, Inc.

Bruner, R.H., E.R. Kinkead, T.P. O'Neill, C.D. Flemming, D.R. Mattie, C.A. Russell, and H.G. Wall. 1993. The toxicologic and oncogenic potential of JP-4 jet fuel vapors in rats and mice: 12-month intermittent inhalation exposures. *Fundam. Appl. Toxicol.* 20:97–110.

Carpenter, C.P., E.R. Kinkead, D.L. Geary, Jr., L.J. Sullivan, and J.M. King. 1975a. Petroleum hydrocarbon toxicity studies: VI. Animals and human response to vapors of "60 solvent." *Toxicol. Appl. Pharmacol.* 34:374.

Carpenter, C.P., E.R. Kinkead, D.L. Geary, Jr., L.J. Sullivan, and J.M. King. 1975b. Petroleum hydrocarbon toxicity studies: VI. Animals and human response to vapors of "70 solvent." *Toxicol. Appl. Pharmacol.* 34:395.

Frith, C.H., B. Highman, G. Burger, and W.D. Sheldon. 1983. Spontaneous lesions in virgin and retired breeder BALB/c and C57BL/6 mice. *Lab. Anim. Sci.* 33:273–286.

Gaworski, C.L., J.D. MacEwen, E.H. Vernot, R.H. Bruner, and M.J. Cowan. 1984. Comparison of the subchronic inhalation toxicity of petroleum and oil shale JP-5 jet fuels. In: *Applied Toxicology of Petroleum Hydrocarbons.* M.A. Mehlman, ed. Vol. 6, pp. 33–47. New Jersey: Princeton Scientific Publishers, Inc.

Gaworski, C.L., C.C. Haun, J.D. MacEwen, E.H. Vernot, R.H. Bruner, R.L. Amster, and M.J. Cowan. 1985. A 90-day vapor inhalation toxicity study of Decalin. *Fundam. Appl. Toxicol.* 5:785–793.

Haseman, J.K., J. Arnold, and S.L. Eustis. 1990. Tumor incidences in Fischer 344 rats: NTD historical data. In: *Pathology of the Fischer Rat.* G.A. Boorman et al., eds. pp. 555–564. New York: Academic Press.

Kinkead, E.R., L.C. DiPasquale, E.H. Vernot, and J.D. MacEwen. 1974. Chronic toxicity of JP-4 jet fuel. In: *Proceedings of the Fifth Annual Conference on Environmental Toxicology,* AMRL-TR-74-125 (ADA-011563), pp. 145–154. Wright-Patterson Air Force Base, OH: Aerospace Medical Research Laboratory.

Kinkead, E.R., C.L. Gaworski, C.D. Flemming, R.H. Harris, W.M. Witt, H. Davis, and R.E. Schmidt. 1991. *Tumorigenic Evaluation of Jet Fuels JP-TS and JP-7.* AL-TR-1991-0020 (ADA-252012). Wright-Patterson Air Force Base, OH: Armstrong Laboratory.

Kinkead, E.R., S.A. Salins, and R.E. Wolfe. 1992. Acute irritation and sensitization potential of petroleum-derived JP-4 jet fuel. *Acute Toxicity Data,* Vol. 11, No. 6, p. 702.

Kinkead, E.R., R.E. Wolfe, and S.A. Salins. 1993. Acute oral and inhalation toxicity of petroleum-derived JP-4 jet fuel. *Acute Toxicity Data,* Vol. 12, No. 6, p. 635.

MacEwen, J.D. and E.H. Vernot. 1975. *Toxic Hazards Research Unit Annual Technical Report*, AMRL-TR-75-57 (ADA-019456). Wright-Patterson Air Force Base, OH: Aerospace Medical Research Laboratory.

MacEwen, J.D. and E.H. Vernot. 1980. *Toxic Hazards Research Unit Annual Technical Report*, AFAMRL-TR-80-79 (ADA-075976). Wright Patterson Air Force Base, OH: Aerospace Medical Research Laboratory.

Mattie, D.R., C.L. Aldren, T.K. Newell, C.L. Gaworski, and C.D. Flemming. 1991. A 90-day continuous inhalation study of JP-8 jet fuel followed by 20 or 21 months of recovery in Fischer 344 rats and C57BL/6 mice. *Toxicologic Pathology* 19:77–88.

Ridder, G.M., E.C. Van Barger, C.L. Alden, and R.D. Parker. 1990. Increased hyaline droplet formation in male rats exposed to Decalin is dependent on the presence of $\alpha_{2\mu}$-globulin. *Fundam. Appl. Toxicol.* 15:732–743.

Swenberg, J., B. Short, S. Borghaff, J. Strasser, and M. Charbonneau. 1989. The comparative pathobiology of alpha$_{2\mu}$-globulin nephropathy. *Toxicol. Appl. Pharm.* 97(1):35–47.

7.2 OVERVIEW OF THE VAPOR GENERATION AND ANALYSIS PARAMETERS OF THE PETROLEUM- AND SHALE-DERIVED FUEL STUDIES CONDUCTED IN THOMAS DOME EXPOSURE CHAMBERS AT THE TOXIC HAZARDS RESEARCH UNIT, WRIGHT-PATTERSON AFB (DAYTON), OHIO, 1973–1983

H. F. Leahy

ABSTRACT

From 1973 to 1983, a series of subchronic, multiple-species, inhalation exposures were performed to evaluate the toxicity of aviation and marine fuels derived from petroleum or shale. The fuels included JP-4, JP-5, JP-7, JP-8, JP-TS, and diesel marine. The inhalation chambers used for these studies were the Thomas Domes. The target total hydrocarbon (TH) concentrations ranged from 0.05 to 5.0 mg/L. The TH vapor concentrations were limited by the vapor pressure of the type of fuel and, if exceeded, the formation of condensate aerosols. The generation and analytical monitoring of the TH vapor exposures are described. A qualitative perspective of the neat fuel, vapor, and waste TH components is provided. Under the conditions defined for these studies, hydrocarbons above C_{14} were in very low concentrations in any of the fuel vapors.

INTRODUCTION

Inhalation toxicology of fuels, solvents, and hydraulic fluids has been an important part of the work at the Toxic Hazards Research Unit over the past 20 years. During this time, there were many changes in the equipment available to describe the exposures, while at the same time, equipment to produce them was only slightly modified. This report will be limited to some of the petroleum and shale fuels tested and will describe the basic methods involved in generation and analysis of the chamber atmospheres and problems encountered in the process. These studies were performed in the Thomas Dome inhalation chambers (Thomas, 1965), which are located in the Armstrong Laboratory at Wright-Patterson Air Force Base. The Thomas Domes are unique exposure chambers adaptable to both altitude and ambient continuous studies of both aerosols and vapors.

ANALYSIS

Mass Composition

All petroleum-and shale-derived fuels are complex mixtures of mainly aliphatic and some aromatic compounds, while the most distinguishing feature is the relative composition of these compounds based on the distillation cut temperatures. The specific mixture is not only influenced by

end use, which determines the physical characteristics such as volatility and viscosity, but the chemical composition is also a function of the source of the crude and the cracking process. Each of the fuels used in these studies was obtained from a single batch of fuel passing the physical requirements for that fuel type.

Quantifying the total petroleum (or shale) hydrocarbon (TPH) of such a complex mixture is possible with the use of a flame ionization detector (FID) adapted for continuous analysis. The FID response to the mixed alkyls is based essentially on the mass concentration of the vapor, independent of the chain length. This fact permitted calibration using known mass concentrations of a convenient hydrocarbon. Beckman 400 hydrocarbon analyzers (Beckman Inst. Co., Fullerton, CA) were used for all TPH analyses. Propane, hexane, and heptane, as well as benzene, have been used as quantifying standards. Hexane has been recommended as a universal standard for TPH calibration.

Initially, the number of hydrocarbon analyzers was limited, and sample dilution was necessary for analysis of two chambers of different concentrations with a single analyzer. Attention to the dilution was essential for accuracy. Later, each chamber, as well as an industrial hygiene system, was equipped with an analyzer.

Component Composition

During the initial studies, gas chromatography was used to determine the benzene concentration of the fuel and the chamber atmosphere. Samples from each of the supply drums were chromatographed and areas of a number of the peaks eluting during the first 20 min were used to compare the supply drums of fuel for quality control.

The use of packed columns, the lack of column oven temperature programming, and no automatic integration limited data acquisition for the first studies. But with the availability in time of increased analytical capability, more information was made available. These data were not always part of the study request but were an attempt to expand analytical capability and to supply quality control information. The study protocols called for a mass concentration without a definitive cut description, while the authors of the protocols assumed the exposure would resemble that which follows a fuel spill. A representative set of the available chromatograms is presented in a Department of Defense (DoD) technical report (Leahy, 1994).

Chromatography Equipment

A Varian 1200 (Varian Inst. Corp., Walnut Creek, CA) with packed columns supplied the information for benzene analysis in studies conducted prior to 1978. Chromatograms were limited to the first 20 min of an isothermal run; a System One computing integrator (Spectra Physics, Santa Clara, CA) was used for some quality control data.

When the Varian 3700 chromatograph (Varian Inst. Corp., Walnut Creek, CA) was obtained and the first temperature programmed chromatograms were developed, the complexity of fuels was first appreciated and the problems of integration were encountered. The System One memory was insufficient for the number of peaks obtained when capillary columns were introduced.

A constant problem for any fuel's chromatography is the overwhelming number of peaks of very similar materials, which results in a rising baseline from both overlapping peaks and temperature programming. Accurate integration of these factors becomes a difficult problem, if not an impossibility. Although hard numbers from very similar integrations are not available from all of the different studies represented here, visualization of some representative chromatograms is of value for comprehension of the fact that the exposures were more alike qualitatively than one might have expected (data not shown).

The acquisition of an HP-5780 computing integrator in 1978 allowed better data analysis with improved integration and the ability to reintegrate and run area slices programs (Figures 7.2-1 and 7.2-2). These demonstrate the significant differences of liquid, vapor, and waste.

Aerosol

Shortly after the start of the petroleum JP-5 study in 1977 (MacEwen and Vernot, 1978), a condensate aerosol caused the loss of many of the mice, necessitating a restart of the study with replacements and at a lower concentration. Cold chamber temperatures, a high vaporization temperature, and low chamber air flow contributed to the problem. Generation conditions were thereafter tested with respect to the potential for an aerosol episode before the start of each new exposure, and target concentrations were scaled downwards if a problem was encountered. Also, routine aerosol counting was introduced using a Model 225 aerosol particle counter (Royco Instruments, Inc., Menlo Park, CA) for the remainder of the fuel studies.

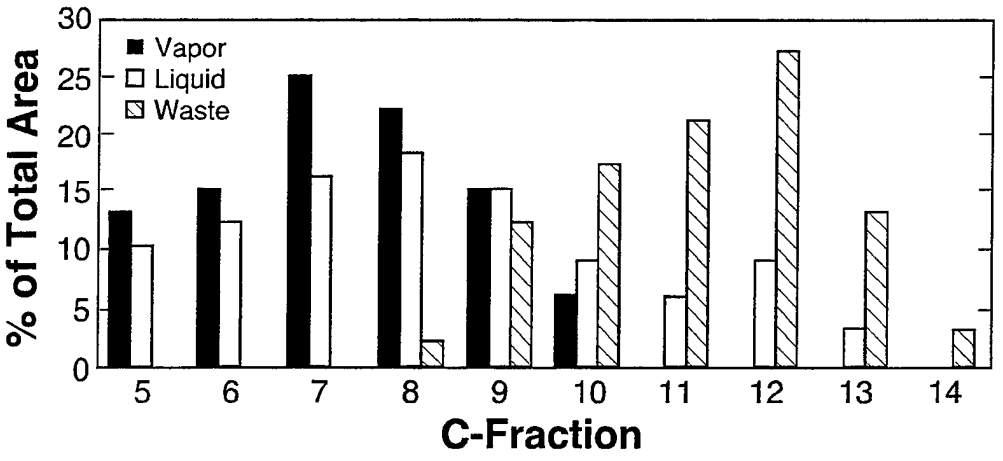

Figure 7.2-1. **Bar graph plot of area slices data for shale JP-4 comparing relative percent of total peak area for vapor, liquid, and waste samples**

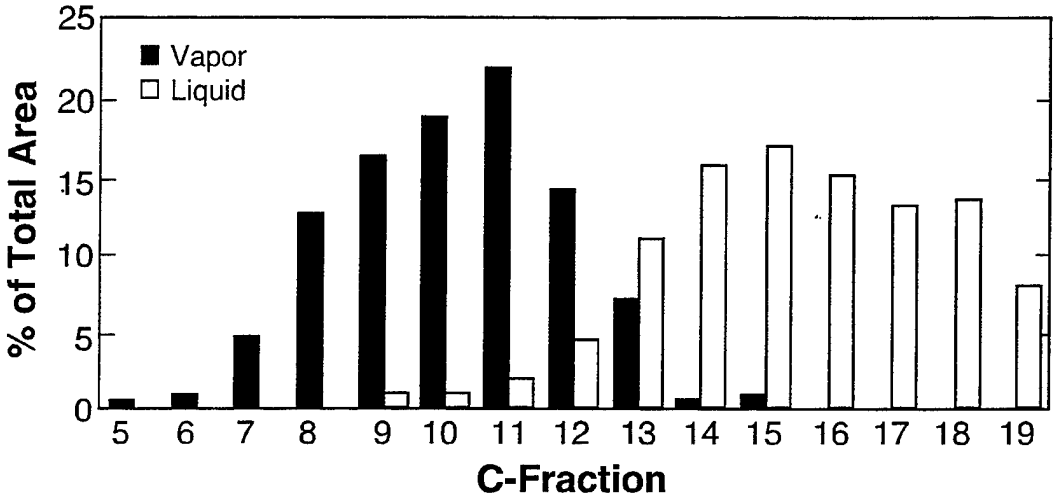

Figure 7.2-2. **Bar graph plot of area slices data for shale DFM comparing relative percent of total peak area for vapor and liquid sample.**

Formation of the condensate aerosol appeared to be a critical event triggered by evaporator temperature that was too high, or chamber temperature that was too low. The only effective way to increase the TPH for the heavier fuels was some combination of an increase of fuel flow and evaporator air supply, thereby supplying more of the lighter chain components for vaporization.

For valid sampling with the Royco particle counter, attention had to be paid to the dome/dome room differential pressure due to the type of sample pump in the instrument.

Target Concentrations

The first study of petroleum-based JP-4 (MacEwen and Vernot, 1974) targeted a specific benzene concentration in the atmosphere as well as the total hydrocarbon present. At that time, the benzene concentration of fuels was significantly higher than any fuels used during subsequent studies. With the 5 and 2.5 mg/L TPH vapor exposures, a 25 and 12.5 ppm exposure to benzene was accomplished. High fuel flow rates (30 to 40 mL/min) and low generator temperatures were combined in this study to favor an increase of benzene in the vapor.

The study on petroleum JP-5 (MacEwen and Vernot, 1978) targeted a 1.5 mg/L TPH concentration with 10 ppm benzene. The fuel not only contained insufficient benzene to attain 10 ppm, but the total quantity of fuel available was limited. Only enough supply was available for use of 10 mL/min/tower for the length of the exposure. The attempt to operate at 1.5 mg/L with the limited flow and a generation temperature about 135 °F ended with an aerosol excursion. The study was restarted with fresh animals at a lower target concentration of 0.75 mg/L.

After experiencing the aerosol problem, the requested TPH concentrations for all further studies were tested and modified when determined necessary. The fuel evaporating temperature was kept below 135 °F and fuel flow rate, as well as evaporator air flow, were increased to raise TPH vapor output. If all else failed, the target concentration was lowered to prevent formation of aerosol.

GENERATION

Evaporation Tower System

The evaporation towers used were common for all of the fuel studies, as well as for a number of solvent and specialty fuel studies where an output of a high volume of vapor was required.

The central zone of the glass tower was a cylinder 13 in. long by 1-3/4 in. O.D. It had a 13-turn spiral, 9 in. long, impressed in the wall to hold a heating coil and lengthen the vaporization path. The top reduced to a "T" with a 1-in. O.D. right arm for vapor exhaust and a 1/4-in. connector for input fuel. The bottom reduced to a 1-in. O.D. glass tubing connected to a double "T" of stainless steel tubing. Pipe fittings at this connection served for waste fuel drain and carrier air input.

Up to three towers were used to generate the total amount of fuel vapor required. To ensure qualitatively comparable exposures in the chambers, the tower outputs were combined in a manifold and routed to the domes by controlled volume flow proportional to the specified dome concentration. Fine chamber concentration control was accomplished during normal operation by minor changes of the individual dome flows.

Some of the heat energy for vaporization was supplied with the incoming air, but most came from an electrically heated coil wrapped around the tower. This was composed of a 1/4-in. close coiled nicrome wire (B&S 20-gauge, 1.1 W/in., Wooge Manufacturing Co., Chicago, IL) approximately 6 ft long. The temperature of the tower output (air and vapor mix) was monitored using probes in the fittings at the top. There was no measure of the internal tower wall temperature. The waste fuel temperature was also monitored.

During the initial studies, the desired output temperature was obtained using 120V AC power rheostats, which supplied a constant voltage to the coil. The efficiency of the vapor output was affected by the temperature of the incoming air as well as that of the vaporization surface area. The input power was manually modified in response to the output temperature. The later studies made use of a proportional temperature controller that continuously monitored the tower output temperature and modified the input power to maintain a set temperature automatically. This eliminated the decreased output from cold temperatures, and actually replaced it with a slight increase of vapor output during cold external conditions.

The input fuel flow rate was controlled with a needle valve and monitored using Fisher & Porter rotameters (Warminster, PA). Fuel was supplied to the system directly from a 55-gallon drum pressurized at 5 to 8 psi. The control valve was positioned after the rotameter to prevent effervescence in the flow tube. The waste fuel flow was partially throttled to reduce the loss of tower vapor output and was pumped from an intermediate catch tank to a waste collecting drum. Redundant supply and waste systems allowed continuous operation while replacing drums.

The generation system was protected from fire by monitoring probes and a solenoid switch set to shut off the fuel flow in case of temperature overrun of either the fuel vapors or the waste fuel.

Evaporation Tower Operation

Except for the first two studies where a specific benzene concentration also was expected, the goal was to supply a specific and constant TPH concentration without creating an aerosol. The conditions required to attain the target concentrations were, in general, established empirically.

The evaporator towers operated as counter flow systems. The fuel entered the top of the towers in a 1/8-in. O.D. teflon tube and the air entered at the bottom, bypassing the spent fuel. The fuel flowed from a point above the heating coil where the tube touched the glass, then down the

sidewall of the evaporator in an uncontrolled stream. As it descended, the composition of both the fuel and the vapors generated were in a dynamic state of change. The upper tower temperature sensor monitored the mixed air and fuel vapor leaving the tower and not that of the evaporating surface area of the tower, while the lower tower sensor monitored the temperature of the spent fuel. Consistent tower operation and pooling of the outputs of two or more towers provided qualitatively similar output for the operation of up to three chambers.

At a specific temperature, the effective vapor pressure of an individual component in a mixture is a function of its vapor pressure times its mole percent concentration multiplied by an activity coefficient of the system. The activity coefficient is related to the solvent, the temperature, and the pressure. For the mixed components of the paraffinic series, this latter effect should be a minor factor due to the similarity of the components (Bishop, 1981). The complexity of the evaporation of the fuel was not only due to the multiplicity of components, but also of its changing percent composition and temperature as it flowed down the tower wall. The composition of vapors obtained was a compromise somewhere between a headspace sample and the original fuel with a significant reduction after C_{13} due to the effectively low vapor pressures of the longer chain hydrocarbons.

In the system as operated, increasing the fuel flow rate, short of initiating splashing, while maintaining air flow rates resulted in a greater TPH output due to the presence of more volatile components available for stripping, as well as from the additional heat supplied to maintain the output temperature of the vapor and air mix. This would favor a relative increase in the front end component of the vapor. An increase of the carrier air flow alone also increased the TPH output by removing more air per unit time while also causing more heat input to maintain the same output temperature. This would shift the vapor to contain somewhat more of the less volatile component.

On the other hand, from the same fuel and air flow, but at a higher operational temperature, the TPH vapor output also could be readily increased. However, as the fuel temperature increases, the vapor pressures of the heavier hydrocarbons increase relatively faster than those of lighter ones, while upon cooling, the reverse occurs, thus contributing to the probability of an oversaturated system, followed by formation of a condensate aerosol. An upper tower temperature of about 135 °F was empirically determined to be near a critical upper limit for operation of the system with acceptable aerosol measurement.

RESULTS

Vapor

The overall effect due to (a) the nature of the fuels (broad distillation cuts), (b) the manner of achieving the target concentrations, and (c) the temperature restriction on vapor generation was to favor whatever lighter fraction was present. The target concentrations for JP-4 were easily met because of the predominance of the light hydrocarbon fraction. In an attempt to achieve the targeted concentration with increased heat due to a limitation of available fuel for the project, the first JP-5 study experienced a condensate aerosol on cooling of the vapors. That study was restarted at a lower target concentration. The most extreme example of the difference of vapor from the parent fuel was the chamber atmosphere produced from shale-derived diesel fuel marine (SDFM), where over 50% of the TPH vapors were from decane (C_{10}) and lower molecular weight components, which had represented less than 2% of the original fuel (Table 7.2-1 and Figure 7.2-2). The volatility restrictions of heavier fuels were overcome by the increased flow rate of fuels through the towers and the lower targeted chamber TPH concentrations. Thus, the exposure atmospheres of both petroleum and diesel, JP-5 and DFM, also JP-7, JP-TS, and JP-8, were more similar qualitatively than would have been expected if considering only the starting fuel composition. A series of chromatograms, where available, qualitatively compared the chamber vapors with input fuels and spent fuels.

TABLE 7.2-1 GAS CHROMATOGRAPHIC AREA SLICE INTEGRATION ANALYSIS COMPARISON OF THE LIQUID SHALE DFM WITH THE CHAMBER VAPOR

	Liquid DFM		Chamber Atmosphere	
Fraction	% of Total Area	Cumulative %	% of Total Area	Cumulative %
< C5	N.I.	—	1.0	1.00
C5	N.I.	—	0.35	1.35
C6	N.I.	—	0.83	2.18
C7	N.I.	—	4.51	6.69
C8	N.I.	—	12.59	19.28
C9	0.90	0.90	16.41	35.69
C10	0.98	1.88	18.60	54.29
C11	1.81	3.69	21.71	76.00
C12	4.22	7.91	14.08	90.08
C13	10.90	18.81	6.93	97.01
C14	15.56	34.37	0.44	97.45
C15	16.77	51.15	0.63	98.08
C16	14.89	66.03	N.I.	—
C17	12.74	78.77	N.I.	—
C18	13.33	92.10	N.I.	—
C19	7.39	99.49	N.I.	—
C20	0.52	100.00	N.I.	—

Note: (a) The fractions (area slices) are designated by the normal alkane number and include all compounds between the previous normal alkane up to and including the designated normal alkane.
(b) N.I. = not integrated.

Fuel Description

With availability of good chromatography and integration, the composition of a complex fuel or fuel vapor can be described graphically or numerically in a simpler form by application of the concepts of simulated distillation and area slicing. This can be accomplished by dividing the chromatogram into logical subunits, in this case by the normal alkanes, and summing the peak areas within the subunits. By plotting on probability graph paper the accumulated percent area of the slices (subunits) against the boiling point of the designated carbon fraction, the temperature at the

50% point as well as temperatures at 16% and 84% can be determined (Figure 7.2-3 and Table 7.2-2). A relatively complete description of the nature of the complex fuel mix will be available with three numbers. These express the 50% cutoff temperature and the temperature spread of the distillation cut of the majority of the components of the fuel.

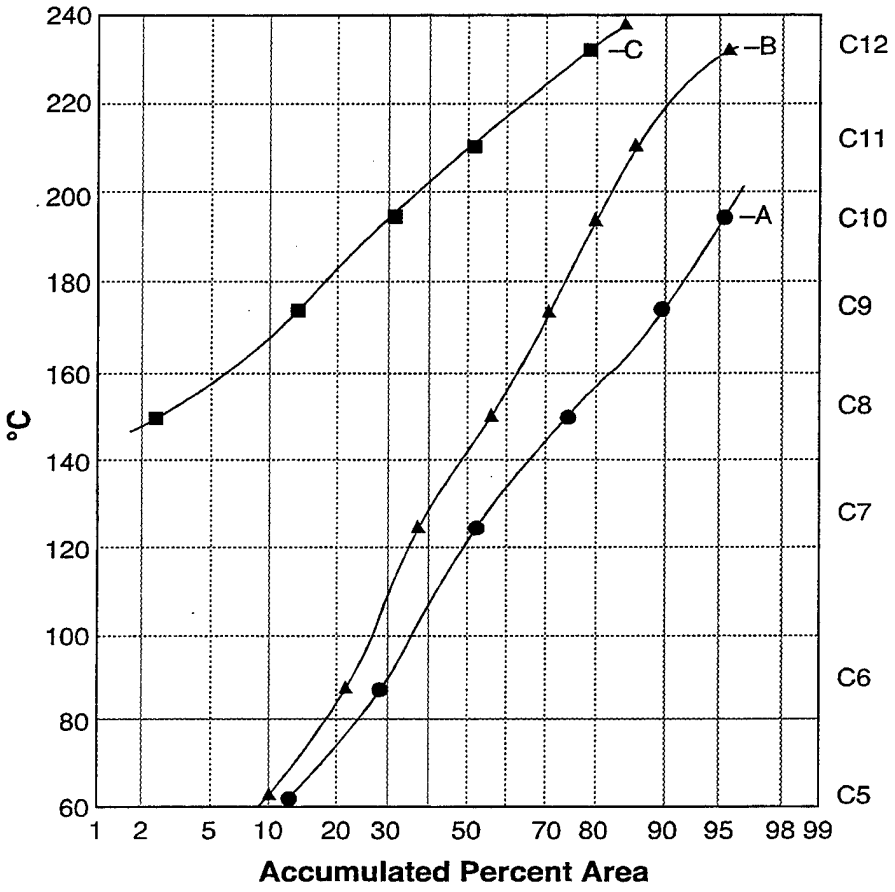

Figure 7.2-3. Probability plot of area slice data to determine the 16, 50, and 84% temperature cut-off points. A = Vapor, B = Liquid, C = Waste of Shale JP-4.

TABLE 7.2-2. SIMPLIFIED FUEL DESCRIPTION USING DATA OF ACCUMULATED PERCENT PEAK AREA FROM SIMULATED DISTILLATION OF SHALE JP-4

	Accumulated %		
Phase	**16%**	**50%**	**84%**
Liquid	73 °C	141 °C	205 °C
Vapor	66 °C	122 °C	161 °C
Waste	176 °C	209 °C	238 °C

Operational Problems

1. Temperature

The input air and fuel temperatures and that of the generation area itself were not completely controlled, having daily as well as seasonal, and sometimes, emergency fluctuations. These temperature fluctuations affected the tower efficiency, especially when the heat input was controlled with a specific rheostat setting and required adjustments to make up for changing conditions.

Variations in fuel viscosity due to temperature shifts affected the rotameter ball position for the same flow rate. Because a specific setting was predetermined for a study, corrections were made to return the ball to that position slightly lowered flow when the fuel was colder and raised it when it was warmer.

During continuous studies, the operating parameters were checked at least hourly, and TPH concentrations were adjusted when outside a range of ± 5% of targeted concentrations. Audible alarms were activated if the concentration exceeded ± 10% of the desired concentration, and immediate attention was given to the problem. Minor adjustments were made using the chamber flow rate within limits rather than the generation system.

2. Splashing

The splashing of fuel at the bottom of the tower became a potential problem whenever the fuel input went above 13 mL/min. The droplets of the spent fuel hitting the hot glass wall in the zone of incoming air changed the composition of the vapor to include more of the heavier diesel range organics. When it occurred, this contributed to (a) the potential for aerosolization on cooling of the vapor, (b) greater similarity of the chamber atmosphere with that of the supply material, and (c) very rapid changes of chamber TPH concentration. A tower air input line modification extended the line above the pool of the spent fuel, thus reducing the probability of splashing from the bottom.

A similar result could have occurred if the inlet line separated from the glass wall and fuel falling through the air stream was blown against the hot glass or blown through as an aerosol. One report of the JP-8 study (Mattie et al., 1991) states that the entire fuel was vaporized. This idea may have resulted from misidentification of samples, sampling problems, or the dropping through of liquid fuel. On searching archived records, it was observed that at times the record showed little difference between the atmosphere and the parent material as judged from the areas of a number of marker peaks. But with the inclusion of more of the available data and the comparison of a number

of chamber vapor chromatograms, it appeared that at times fuel splashing or dropping through had occasionally occurred. However, most of the time the vapor was composed as in the other studies favoring the more volatile components. As a method for getting a vapor more representative of the fuel itself, this phenomenon might deserve study.

3. Sampling

The Beckman 400 hydrocarbon analyzers were operated continuously. Samples were drawn from the chambers which operated at 20 mmHg negative to ambient, through 1/4 in. stainless steel lines, and pressurized by diaphragm pumps (Diapump, Model 08-800-70, Air Control Inc., Norristown, PA) to about 3 psi with flow about 3 to 4 L/min. Calibration was performed before each of the studies, and routine calibration checks were performed during the studies. The analyzers performed very well for extended periods without significant drifting of output signal. One early problem was a bleed-off of hydrocarbons from the pump check valves. This was corrected when each dome analytical system became independent and baseline air was delivered with a separate pump.

4. Chromatography

Headspace samples were used initially for determination of the benzene content. They were convenient and reasonably reproducible while serving the purpose intended. They also provided a method for quality control.

Dilution in hexane permitted an injection of a minute sample of the fuel presenting the first full chromatograms. But the presence of the hexane masked a problem encountered where approximately one-third of the barrels (the first five received) of shale JP-5 contained 3% aviation gasoline (AvGas), a contaminant from a tank at Rickenbacker Air Force Base. Even in the less contaminated samples from the rest of the supply, there was enough AvGas to cause a bimodal distribution in the vapor phase (data not shown).

Delivering a valid vapor sample was a problem with the back pressures of the old packed column systems while the very low concentration studies created problems for the capillary systems. Generator output samples were sometimes invalid because of contamination with splashed or condensed fuels. Some apparent discrimination in TPH component distribution could be caused due to incomplete vaporization from the needle or the injection port. Generator output samples were potentially contaminated by recondensing vapors at the sample port. These required judgment whenever there was disagreement with the chamber samples. Their advantage was that the concentration was much higher and the chromatograms more reliably integrated because they had flatter baselines.

Even though the chromatography data were from a variety of equipment, the data presented a consistent picture of all but the first few exposures. The JP-4 studies, shale and petroleum, differed somewhat from the rest with a significantly higher concentration of the front-end components, these being readily available in the parent mixture. The rest showed more similarity of the vapor phase to one another than would have been expected from differences in the liquid if not considering the limitations imposed by the component vapor pressures (Table 7.2-3).

TABLE 7.2-3. AN ESTIMATION OF THE DISTRIBUTION OF THE MAJOR ALKANE FRACTIONS PRESENT IN THE THREE PHASES OF THE FUELS

Fuel Type	Liquid	Vapor	Waste
P-JP-4	C7-C15	C6-C8	NA
S-JP-4	C5-C9-C13	C5-C9	C10-C14
P-JP-5	C10-C13	NA	NA
S-JP-5A	C6-C8-C13	C6-C7/C10-C11	C10-C13
S-JP-5	C10-C13	C10-C11	C10-C13
P-DFM	C11-C18	NA	NA
S-DFM	C11-C18	C8-C12	C11-C18
JP-7	C11-C14	C10-C12	NA
JP-TS	C9-C13	C9-C11	NA
JP-8	C9-C12	C9-C10	NA

Note: NA = not available

The shale JP-5 contaminated with AvGas actually had a bimodal distribution of components in the vapor phase with all but complete stripping of the front-end materials from the fuel. The shale-derived JP-4 also appeared bimodal, but not as severely as the contaminated shale JP-5. There were some similarities in shale JP-4 front-end and petroleum JP-4. This could have been the result of a similar cracking process, contamination, or probably blending to achieve the required physical characteristics for a JP-4 type of the fuel containing significantly greater amounts of the lower fractions.

Operational Parameters

Many factors were involved in the choice of dome and generation flows. Some of these were: (a) a benzene requirement; (b) limited fuel supply for the study; (c) minimal allowable dome flow; and (d) fielder's choice of the chemist within these restrictions. Details of some of the basic operational parameters for select JP studies are presented in a DoD technical report (Leahy, 1994).

REFERENCES

Bishop, Edward C. 1981. Evaluating Health Hazards Associated With Aircraft Fuel Cell Maintenance. AFAMRL-TR-81-149, Proceedings of the Twelth Conference on Environmental Toxicology, 3–5 November 1981.

Leahy, H.F. 1994. Overview of the vapor generation and analysis parameters of the petroleum- and shale-derived fuel studies conducted in Thomas Dome exposure chambers at the Toxic Hazards Research Unit Wright-Patterson AFB (Dayton), Ohio, 1973–1983 (in press).

Mattie, D.R., C.L. Alden, T.K. Newell, C.L. Gaworski, and C.D. Flemming. 1991. A 90-day continuous vapor inhalation toxicity study of JP-8 jet fuel followed by 20 or 21 months of recovery in Fischer 344 rats and C57BL/6 mice. *Toxicol. Pathol.* 19, 77.

MacEwen, J.D., and E.H. Vernot. 1974. Toxic Hazards Research Unit Annual Technical Report: 1974. AMRL-TR-74-78. pp. 5–26.

MacEwen, J.D., and Vernot, E.H. 1978. Toxic Hazards Research Unit Annual Technical Report: 1978. AMRL-TR-78-55. pp. 49–71.

Thomas, A.A. 1965. Low ambient pressure environments and toxicity. *Arch. Environ. Health* 11, 316–322.

8.1 STATISTICAL DESCRIPTION FOR SEVEN NAVAL POPULATIONS

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INTRODUCTION

This project explores the effects of considering specific military subpopulations in estimating the potential for chemical toxicity. Populations of interest may be identified due to differences in exposure characteristics or differences in response to an exposure dose. In some cases, such as chemicals in drinking water, the populations exposed are quite varied. In other cases, exposures occur for specific groups that are actively using a chemical or closely associated to such use. Civilian or military workers would be an example of this latter case.

Physiological variations can also define populations of interest. These variations may affect the absorption, distribution, metabolism, or elimination (pharmacokinetics) of a chemical, or the mechanism of toxicity (pharmacodynamics) of a chemical. Evaluation of differences in pharmacokinetics using physiologically based pharmacokinetic models (PBPK) is the focus of this study.

The general choices for considering military subpopulations included known population samples and characteristics of SEALS (SEa, Air, Land), divers, aviators, and fleet sailors. The SEALS and divers were believed to be a trimmer, more athletic community. The aviators were known to have stringent flight physicals they were required to pass periodically. The fleet sailors were chosen to provide a benchmark for comparisons and represent most of the active duty personnel.

The results of this project will be used to describe the statistical distribution of each subpopulation and perform Monte Carlo simulations on the subpopulations to evaluate differences in pharmacokinetics. Another use of the results will be to aid the Navy in revising physical fitness standards.

METHODS

Collection of Data

The populations and sample sizes used in this report were largely dependent upon the availability of data from the various communities within the Navy. All data were obtained from various Naval research/data holding facilities.

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Sample sizes were chosen to avoid statistical inadequacy. In selecting the number from each community there was no intention of having a "proportionate sample" of the communities in the Navy. For mixed-sex populations, aviators and fleet, every effort was made to ensure that females were adequately represented.

Outliers

Through descriptive statistics (minimum and maximum), the potential for outliers was evaluated among the Naval populations. Age, body fat, body weight, and body height were correlated; hence, a multivariate method of outlier identification was used. The method was principal component analysis. The method determines factors (axes) that represent the correlational structure of the original variables (Barnett, 1984). The Mahalanobis distances from each case to the centroid (mean of age, mean of body fat, mean of body weight, mean of body height) for the original data is computed. Also, the Mahalanobis distances from each case to the factor scores is computed. The difference between the above distances is computed. Distances have a chi-squared distribution for large samples. If the chi-squared for the difference is significant ($p < 0.01$), the case is considered an outlier. An outlier was eliminated if it was an obvious data entry error.

Estimate the Distribution for Each Population

The determination of a distribution requires measuring the following components: central tendency (mean and median), variation (standard deviation, minimum, and maximum), correlation among the variables (correlation and covariance), and the theoretical distribution (normal, log-normal, and others). The distributional properties of body fat (%), body weight (kilograms), and body height (centimeters) were calculated for each population. The Wilk-Shapiro ($5 \leq n \leq 2000$) test of normality (Dixon, 1990) or the skewiness-kurtosis chi-squared test of normality ($n > 2000$) were used to test the normality of the data or the log of the data.

Modeling the Correlational Structure of Variables

The Pearson Product Moment Correlation Coefficients were found for the following variables: age (years), body fat (BF), body weight in kilograms (BWKG), and body height in centimeters (BHCM). A casual model was developed and tested. The statistical method used was structural equations (Bentler, 1992).

Equality of Populations

The equality of population means was tested using a one-factorial (populations) multivariate analysis of variance on body fat, body weight, and body height. The assumption of equality of population variances was tested using a Levene's multivariate test for variances. If the Levene's test was significant ($p < 0.05$), Box-Cox transformations were applied to the data. A one-factorial multivariate analysis of variance was applied to the transformed data.

RESULTS

Outliers

Since the data was generated by other laboratories within the Navy, there was a need to identify recording errors. When the statistical multivariate test of outliers (principal component analysis) was applied to each population, outliers were identified. Table 8.1-1 gives the sample size with the original sample size in parentheses. The percent column indicates the percentage of the sample size which were outliers, but the only outliers that were removed were obvious typographical mistakes. The other type of outliers were not removed since there was no scientific reason for removal. The female naval fleet had one outlier (1.6) which was an obvious typographical mistake because the rest of the data were rounded to the nearest whole number. The aviator data was reduced since the body fat was missing.

TABLE 8.1-1. OUTLIERS RESULTS FOR EACH POPULATION ($p < 0.01$)

	Sample Size	Percent
BUDS	39	3
SEALS	48	4
Male Aviators	150(192)[a]	7
Female Aviators	38(58)[a]	11
Male Fleet	2411	8
Female Fleet	317(318)[b]	8
Divers	144	8

a = Original sample size. Sample size was reduced because there were missing data.
b = Original sample size. Sample size was reduced because there was an outlier which was an entry error.

Determine the Distributions of the Population

The body fat (%) was either normally distributed (BUDS, male aviators, female aviators, and male fleet) or log-normally distributed (SEALS and female fleet) (Table 8.1-2). The SEALS showed a different distribution for body fat than the BUDS, who are trainee SEALS. The SEALS increase their body fat to prepare for a mission because a meal schedule during the mission is unknown. The body weight (kg) was either normally distributed (BUDS, SEALS, male aviators, and female aviators) or log-normally distributed (male fleet and female fleet) (Table 8.1-3). The body height (cm) was normally distributed for all the military populations (Table 8.1-4).

TABLE 8.1-2. DISTRIBUTION TEST FOR BODY FAT (%)

	Distribution	Statistic	p-value
BUDS	Normal	0.96	0.24
SEALS	Log-Normal	0.97	0.40
Male Aviators	Normal	0.98	0.18
Female Aviators	Normal	0.96	0.20
Male Fleet	Normal	2.68(2)[a]	0.26
Female Fleet	Log-Normal	0.98	0.29
Divers	Normal	0.97	0.16

a = Skewiness-kurtosis test of normality using a chi-squared (degrees of freedom).

TABLE 8.1-3. DISTRIBUTION TEST FOR BODY WEIGHT (kilograms)

	Distribution	Statistic	p-value
BUDS	Normal	0.99	1.00
SEALS	Normal	0.96	0.13
Male Aviators	Normal	0.98	0.61
Female Aviators	Normal	0.96	0.12
Male Fleet	Log-Normal	$1.96(2)_a$	0.37
Female Fleet	Log-Normal	0.99	0.78
Divers	Normal	0.98	0.22

a = Skewiness-kurtosis test of normality using a chi-squared (degrees of freedom).

TABLE 8.1-4. DISTRIBUTION TEST FOR BODY HEIGHT (centimeters)

	Distribution	Statistic	p-value
BUDS	Normal	0.98	0.86
SEALS	Normal	0.98	0.79
Male Aviators	Normal	0.97	0.06
Female Aviators	Normal	0.96	0.18
Male fleet	Normal	5.90(2)[a]	0.05
Female fleet	Normal	2.73(2)[a]	0.25
Divers	Normal	0.98	0.66

a = Skewiness-kurtosis test of normality using a chi-squared (degrees of freedom).

The age ranges for the Naval populations seem to reflect the amount of training required for the population and to reflect the amount of physical fitness required for the population.

TABLE 8.1-5. AGE (years) BASIC STATISTICS

	Mean	Median	Std. Dev.	Minimum	Maximum
BUDS	22.00	21.00	2.40	19.00	28.00
SEALS	26.00	25.00	4.50	19.00	36.00
Male Aviators	32.00	30.00	7.60	21.00	51.00
Female Aviators	28.00	26.00	6.30	21.00	45.00
Male Fleet	30.00	28.00	7.30	18.00	56.00
Female Fleet	29.00	28.00	6.20	18.00	50.00
Divers	25	24	4.3	18	37

The body fat means divided into three groups: (i) BUDS, (ii) SEALS, male aviators, male fleet, and (iii) female aviators, female fleet. In body fat, the average distance from the mean (standard deviation) differed significantly among the subpopulations.

TABLE 8.1-6. BODY FAT (%) BASIC STATISTICS

	Mean	Median	Std. Dev.	Minimum	Maximum
BUDS	10.44[abcde]	10.85	2.15	5.44	15.23
SEALS	14.18[ce]	13.51	3.44	8.57	25.98
Male Aviators	14.56[cde]	14.45	4.47	2	30
Female Aviators	22[d]	22	6	10	36
Male Fleet	16	16	5.39	1	33
Female Fleet	24[c]	24	5.57	10	44
Divers	12.78[cde]	13	3.68	4	23

a = Significantly different than SEALS at $p < 0.05$.
b = Significantly different than male aviators at $p < 0.01$.
c = Significantly different than female aviators at $p < 0.01$.
d = Significantly different than male fleet at $p < 0.01$.
e = Significantly different than female fleet at $p < 0.01$.

The body weight means separated into two groups: (i) BUDS, SEALS, male aviators, male fleet and (ii) female aviators, female fleet. The body weight variances were not equal among the populations.

TABLE 8.1-7. BODY WEIGHT (kilograms) BASIC STATISTICS

	Mean	Medium	Std. Dev.	Minimum	Maximum
BUDS	76[ce]	76	5.74	63	91
SEALS	79[ce]	79	7.94	67	103
Male Aviators	81[ce]	81	9.17	54	86
Female Aviators	61.5[d]	61.5	7.87	48	83
Male Fleet	81[e]	79	12.21	46	143
Female Fleet	62	61	8.66	41	96
Divers	77.33[ce]	76.43	8.14	58.97	98.2

a = Significantly different than SEALS at $p < 0.05$.
b = Significantly different than male aviators at $p < 0.01$.
c = Significantly different than female aviators at $p < 0.01$.
d = Significantly different than male fleet at $p < 0.01$.
e = Significantly different than female fleet at $p < 0.01$.

The body height means separated into two groups: (i) BUDS, SEALS, male aviators, male fleet and (ii) female aviators, female fleet. The variances for the body height were not equal among the populations.

TABLE 8.1-8. BODY HEIGHT (centimeters) BASIC STATISTICS

	Mean	Medium	Std. Dev.	Minimum	Maximum
BUDS	177.7[ce]	177.8	6.3	165.1	192.4
SEALS	177.7[ce]	176.5	5.1	161.9	188.5
Male Aviators	180[ce]	180	6.0	163	193
Female Aviators	166[d]	165	6.6	155	180
Male Fleet	178.2[e]	177.8	7.3	152.4	203.2
Female Fleet	164.9	165.1	6.7	149.9	184.2
Divers	175.9[bcde]	175.26	6.05	161.29	195.58

a = Significantly different than SEALS at $p < 0.05$.
b = Significantly different than male aviators at $p < 0.01$.
c = Significantly different than female aviators at $p < 0.01$.
d = Significantly different than male fleet at $p < 0.01$.
e = Significantly different than female fleet at $p < 0.01$.

Correlational Structure of Variables

For the BUDS, body weight was correlated with body fat and highly correlated with body height. For the SEALS, age was correlated with body fat, and body weight was highly correlated with body fat and body height. For the male aviators, age was highly correlated with body fat, and body weight was highly correlated with body fat and body height. For the female aviators, body fat was highly correlated with body weight. For the male fleet, body weight was highly correlated with age, body fat and body height; body fat was highly correlated with age and was correlated with body height. For the female fleet, body weight was highly correlated with body fat and body height.

TABLE 8.1-9. PEARSON CORRELATION COEFFICIENTS FOR BUDS

	Body Fat	Body Weight	Body Height
Age	0.3120	0.1679	0.0822
Body Fat		0.3194[a]	0.0196
Body Weight			0.6718[b]

a = Significant at $p < 0.05$.
b = Significant at $p < 0.01$.

TABLE 8.1-10. PEARSON CORRELATION COEFFICIENTS FOR SEALS

	Body Fat	Body Weight	Body Height
Age	0. 2919[a]	0.0926	0.0602
Body Fat		0.6301[b]	0.1034
Body Weight			0.5560[b]

a = Significant at $p < 0.05$.
b = Significant at $p < 0.01$.

TABLE 8.1-11. PEARSON CORRELATION COEFFICIENTS FOR MALE AVIATORS

	Body Fat	Body Weight	Body Height
Age	0.3102[a]	0.0589	0.0159
Body Fat		0.5536[a]	0.0990
Body Weight			0.5132[a]

a = significant at $p < 0.01$.

TABLE 8.1-12. PEARSON CORRELATION COEFFICIENTS FOR FEMALE AVIATORS

	Body Fat	Body Weight	Body Height
Age	−0.1520	−0.1108	0.0913
Body Fat		0.6992[a]	−0.1955
Body Weight			0.2469

a = Significant at $p < 0.01$.

TABLE 8.1-13. PEARSON CORRELATION COEFFICIENTS FOR MALE FLEET

	Body Fat	Body Weight	Body Height
Age	0.2934[a]	0.1417[a]	0.0224
Body Fat		0.6642[a]	0.0452[b]
Body Weight			0. 5459[a]

a = Significant at $p < 0.01$.
b = Significant at $p < 0.05$.

TABLE 8.1-14. PEARSON CORRELATION COEFFICIENTS FOR FEMALE FLEET

	Body Fat	Body Weight	Body Height
Age	0.0519	0.0506	-0.0151
Body Fat		0.6420 [a]	-0.0235
Body Weight			0.5451 [a]

a = Significant at $p < 0.01$.

TABLE 8.1-15. PEARSON CORRELATION COEFFICIENTS FOR DIVERS

	Body Fat	Body Weight	Body Height
Age	0.2006 [a]	0.1503	0.1123
Body Fat		0.5867 [b]	-0.0770
Body Weight			0.5175 [b]

a = Significant at $p < 0.05$.
b = Significant at $p < 0.01$.

DISCUSSION

In conclusion, age and body height is generally not used in a physiologically based pharmacokinetic (PBPK) model, but the data indicated the need of those variables to be considered. No attempt should be made to incorporate the age and body height in the PBPK models itself. The Monte Carlo simulation of body fat and body weight which are in the PBPK models should be found using the multivariate normal distribution. For the variables of the subpopulations which are log-normal, take the logarithm and apply the multivariate normal distribution to the data.

Since body fat and body weight were correlated with age, the Navy should consider age and body height dependent body fat standards. When age increased, the body fat had a tendency to increase. Since most of the participants in the study were still in the Navy, it would imply that the increase in body fat did affect the performance of an individual.

The study indicated that the lowest to highest body fat was the trainee, male Navy personnel, and female Navy personnel. The female Navy personnel had a body weight of about 62 kilograms; whereas, the male Navy personnel had a body weight in the high 70 kilogram to low 80 kilogram range. The female personnel body height was about 10 cm centimeters lower than male personnel.

REFERENCES:

Barnett, V. and T. Lewis. 1984. *Outliers in Statistical Data*, Second Edition. New York: John Wiley & Sons.

BBN Software Products Corporation. 1988. *RS/1 User's Guide*, Version 4. Cambridge, MA.: BBN Software Products Corporation.

Bentler, P.M. 1992. *EQS: Structural Equations Program Manual*. Los Angeles: BMDP Statistical Software.

Dixon, W.J., M.B. Brown, L. Engelman, and R. I. Jennrich. 1990. *BMDP Statistical Software Manual*, Volume 1. Berkeley: University of California Press.

SAS Institute Inc. 1990. *SAS Procedures Guide*, Version 6. Cary, NC: SAS Institute Inc.

SAS Institute Inc. 1990. *SAS/STAT User's Guide*, Version 6. Cary, NC: SAS Institute Inc.

9.1 1994 CONFERENCE ON TEMPORAL ASPECTS IN RISK ASSESSMENT FOR NONCANCER ENDPOINTS

L. A. Doncaster

The Conference on Temporal Aspects in Risk Assessment for Noncancer Endpoints was held at the Hope Hotel and Conference Center, Wright-Patterson Air Force Base, OH, from 18 through 20 April 1994. The conference was sponsored by the Toxicology Division, Occupational and Environmental Health Directorate, Armstrong Laboratory; the Naval Medical Research Institute Detachment (Toxicology); the U.S. Army Medical Research Detachment of Walter Reed Army Institute of Research; the Office of Research and Development, United States Environmental Protection Agency; and the Division of Toxicology, Agency for Toxic Substances and Disease Registry, with the cooperation of the National Research Council Committee on Toxicology; and was coordinated by ManTech Environmental Technology, Inc., Toxic Hazards Research Unit.

The goals of the conference were (1) recognition of temporal issues in the risk assessment process; (2) examination of critical time factors in hazard identification, dose–response, and exposure characterization; and (3) exploration of limitations in risk characterization.

Session I of the conference previewed the conference themes, namely, applications, including the integration of scientific advances into the protection of human health, and basic research approaches, including scientific examples for addressing temporal issues.

Session II dealt with temporal factors of exposure in identifying hazards and included discussions of critical periods of exposure; timing and duration of exposure; the reversibility of effects; temporal factors affecting immunologic, neurologic (e.g., cholinesterase inhibitors), and reproductive systems; and temporal effects of inhaled vapors and particles on upper and lower respiratory tract and the implications for human health risk assessment.

Session III concerned the effects of exposure patterns on dose–response relationships. Discussions included the definition of dose; the effect of multiple route exposure patterns and the use of external markers and internal markers to determine dose; and the incorporation of exposure patterns in dose extrapolation across time. The final paper of this session included a summary of considerations and concerns on this issue.

The subject of Session IV was exposure assessment and the life cycle timeline. Included were discussions on bioaerosols and the exposure assessment during routine operations; histopathology, now and in the future, as the primary means for identifying male reproductive effects; the Kuwait oil well fires and risk characterization during emergency response; national priority list (NPL) site exposure assessment; and refinements in the exposure assessment process.

Session V addressed characterization of risk in a temporal context. Topics discussed included the assessment of health risk to occupationally exposed Navy personnel; time-response profiles of noncarcinogenic effects in the respiratory tract; temporal aspects of risk characterization for pesticide exposures; and pediatric lead toxicity—temporal aspects of risk communication.

Evening poster and database sessions provided additional scientific information exchange in an informal manner. There were 21 oral presentations, 31 poster presentations, 10 database presentations, and 209 participants in the three-day conference. The conference was highly rated by the participants. Proceedings of the conference will be published in *Inhalation Toxicology*. In addition, the full proceedings, including abstracts of the poster presentations, are being compiled for submission to the Air Force as a technical report.

9.2 1994 HAZMAT/POLLUTION PREVENTION SYMPOSIUM

L. A. Doncaster

The 1994 HazMat/Pollution Prevention Symposium was held at the Hope Hotel and Conference Center, Wright-Patterson Air Force Base, OH, 21 and 22 April 1994. The symposium was sponsored by the Acquisition Environmental Management Directorate, Aeronautical Systems Center, and was coordinated by ManTech Environmental Technology, Inc., Toxic Hazards Research Unit.

The symposium consisted of three sessions. The introductory session included remarks by Lt Gen James A. Fain, Jr., Commander of the Aeronautical Systems Center; Col Joe B. Hollingsworth, Director of Acquisition Environmental Management; and by Mr. Gary Vest, Principal Assistant Under Secretary of Defense for Environmental Security.

Session II addressed pollution prevention program management from the perspective of the Air Force and contractors, and included presentations on the Pollution Prevention Handbook; the C-17 HazMat Elimination Plan; the T-1A Program HazMat Usage Baseline Study; the Northrop B-2 Pollution Prevention Program; the McDonnell Douglas Aerospace—East (MDA-E) Strategic Planning for Pollution Prevention; and the qualification of new cleaning alternatives in aircraft maintenance and overhaul as seen from the perspective of the Environmental Management Division of Lockheed.

The last session was a technical session and discussed material substitutions, again from the perspective of the Air Force and defense contractors.

An evening reception provided the opportunity for interaction between the military and contractors in an informal manner. There were 12 oral presentations in the two technical sessions, and 147 participants in the two-day symposium. A proceedings, consisting of the program and copies of handouts/slides of various presenters, was compiled by the Aeronautical Systems Center.

9.3 1995 CONFERENCE ON RISK ASSESSMENT ISSUES FOR SENSITIVE HUMAN POPULATIONS

L. A. Doncaster

Planning was initiated in September 1994 for the 1995 toxicology conference, "Conference on Risk Assessment Issues for Sensitive Human Populations." It will be held 25 through 27 April 1995 at the Hope Hotel and Conference Center at Wright-Patterson Air Force Base, OH. The conference will be sponsored by Tri-Service Toxicology, Wright-Patterson Air Force Base; the Office of Research and Development, United States Environmental Protection Agency; and the Division of Toxicology, Agency for Toxic Substances and Disease Registry. The planning committee for the conference includes representatives from each sponsoring agency and the Toxic Hazards Research Unit (THRU).

The goals of the conference are (1) identification of sensitive populations in the risk assessment process; (2) understanding biological differences and species variability; and (3) examination of the mechanisms for dealing with sensitivities in hazard identification, dose response, and exposure characterization.

The THRU's Work Plan, which designated a conference coordinator and described invitation and registration procedures, publication procedures, technical support, and the administration of continuing education credits, was submitted to and approved by the Contract Technical Monitor.

Initial conference announcements have been placed in four major scientific journals and a "Call for Papers" flyer has been mailed to over 2700 individuals.

10.1 RESEARCH ENGINEERING SPECIAL PROJECTS

D.L. Courson, H.F. Leahy, W.J. Malcolm, and W.B. Sonntag

The Toxic Hazards Research Unit (THRU) Research Engineering staff has provided technical assistance on a number of research and special projects. This report describes the devices, instruments, and systems that were designed, fabricated, and applied to support several THRU projects. In addition to performing routine maintenance of specialized research systems at the THRU, major renovations of existing systems were undertaken to address efficiency and safety of operation. Additional systems that were developed by the Research Engineering group are described in separate sections of this report.

Design and Construction of a Combustion Atmosphere Inhalation Exposure System.

The THRU Research Engineering group was tasked to design and construct a combustion atmosphere exposure system. This exposure system is being used to evaluate the combustion toxicity of specific materials and examine endpoints of combustion product insult. The exposure system was designed to gather data on settling velocities and rates of dispersal of materials. Original plans were to examine these endpoints at 5, 10, and 15 mph wind speeds in an exposure tunnel. Attempts to imitate 10 mph (880 ft^3/minute) wind speed were unsuccessful using a 12.5 in. duct. After a number of unsuccessful attempts at lower wind speeds, 12 ft^3/min was selected. Exposures are being performed at 12 ft^3/min wind speed.

The exposure system was originally constructed in Building 433, but because of facility constraints, the system was dismantled and rebuilt in Building 824. Utilities had to be modified and/or upgraded to accommodate the exposure system in the new location. Problems with heat dissipation resulted in a redesign of the system. Sixty feet of ductwork were added to the system to allow for accurate measurement of settling velocities of the combustion products. A Spencer turbine blower provided vacuum to the system. Lovelace impactors, particle collectors for electron microscopic sizing, Pitot tubes, and filter samplers were placed in the system to accurately evaluate particle size and distribution. An electrostatic precipitator and gas analyzers were added to provide additional quantitative and qualitative analyses.

Design and Construction of a Nose-Only Inhalation Exposure Facility.

The THRU Research Engineering group was tasked to design and construct benches to support Cannon-designed nose-only inhalation chambers (Cannon, 1983). Two portable benches (4 ft × 7 ft × 2.5 ft) were constructed to support 4 chambers (2 chambers on each bench, Figure 10.1-1). Each Cannon chamber was contained within a plexiglas enclosure with separate exhaust systems (Figure 10.1-2). The benches were equipped with air flow and electrical power for the generation and monitoring of test chemical atmospheres. The exhaust from each chamber was scrubbed through a charcoal bed prior to entering the building exhaust system.

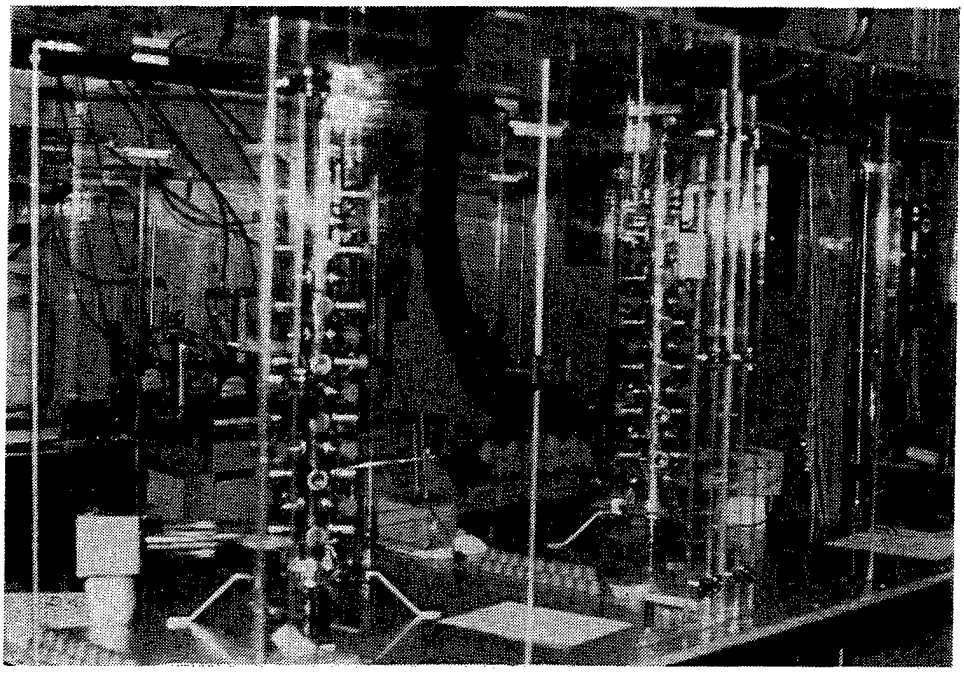

Figure 10.1-1 Nose-Only Inhalation Facility. Bench supporting two Cannon chambers, each within a plexiglas enclosure.

Because rodents are confined within a plexiglas tube for long periods of time (2 to 6 h), temperature within the enclosure area must be maintained at a level that will not result in additional stress to the animals. To provide cooling air to the chambers, a Koldewave (Heat Exchangers, Inc. Skokie, IL) air conditioning system was installed. This system provided cool air using water for heat transfer, thereby not adding additional heat to the laboratory area. Output from the cooler was directed to the four Cannon enclosures. Temperatures within the enclosures have been maintained between 64 ° and 70 °F.

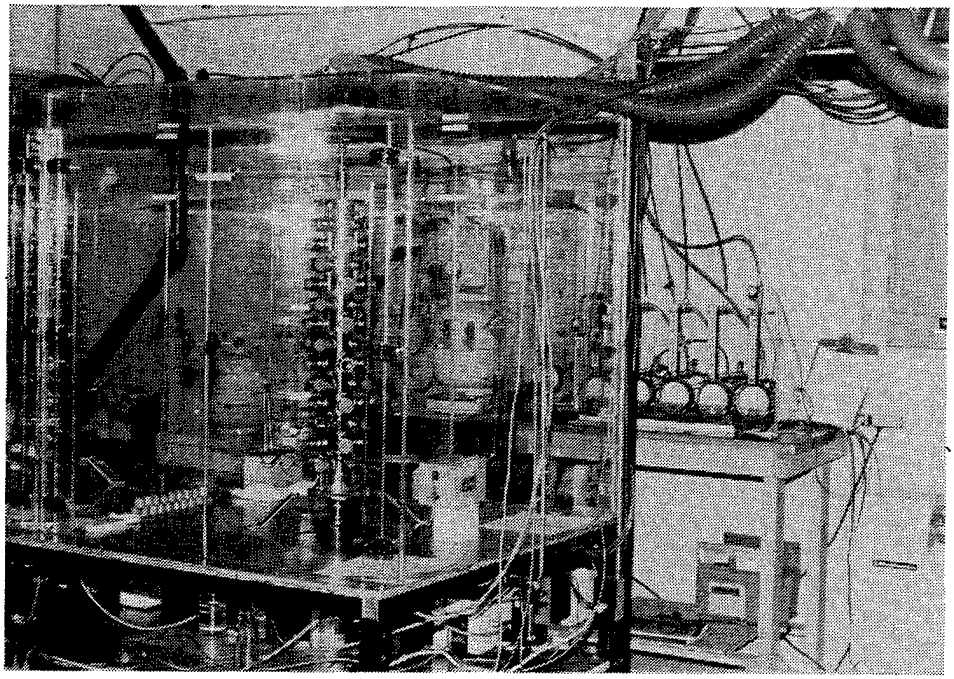

Figure 10.1-2. Nose-Only Inhalation Facility. Plexiglas enclosed chamber showing input and exhaust air lines.

Porous Biomatrix Coating Vacuum Filtration Unit

In response to a request from Armstrong Laboratory, Toxicology Division (AL/OET), a vacuum filtration unit was designed and fabricated to allow the coating of nylon mesh with Biomatrix material without blockage of the mesh pores (Figure 10.1-3). The purpose of this *in vitro* system is to allow attached primary cells access to growth media. This is critical for co-culture experiments when studying biochemical effector molecules released from chemically exposed cells of one type and measuring effects on a second cell type.

Several unique features were required. The unit and components had to be autoclavable, and the Lexan three-piece unit required a combined height of 2.625 in. and an outside diameter of 3.5 in. The outer Biomatrix vacuum chamber had an inside reservoir height requirement of 1.812 in. and a diameter of 3.065 in. The inner coating chamber required a unique canal system containing 3/32 in. diameter slots and circles, with a 2 mm depth to hold the nylon mesh, and a port to the outer chamber. The top Lexan held the mesh in place making for an airtight system with a septum for easy access. Three chambers were fabricated with one chamber serving as a backup.

Small Volume Reagent Reservoir

AL/OET requested the THRU to design and fabricate reagent reservoirs to hold various reagents for biochemical assays using multi-channel pipetters. With the development of more *in vitro* toxicity testing, biochemical assays will be scaled down to accommodate the smaller sample volumes. This is especially true in light of recent developments in fluorescent biomarkers for toxicity.

The newly designed reservoir will allow for rapid transfer of reagents to multi-well plates (Figure 10.1-4). This will result in time saving and more accurate experimental results. The reservoir were formed by three "V-shaped" groves within a block of Lexan plastic that could be autoclaved. The V-shaped grooves were 3 mm wide at the base, and 6 mm deep. Two lengths were designed to accommodate the "6" or "8" pipette tips of a multi-channel pipetter. A lid was designed to protect the base.

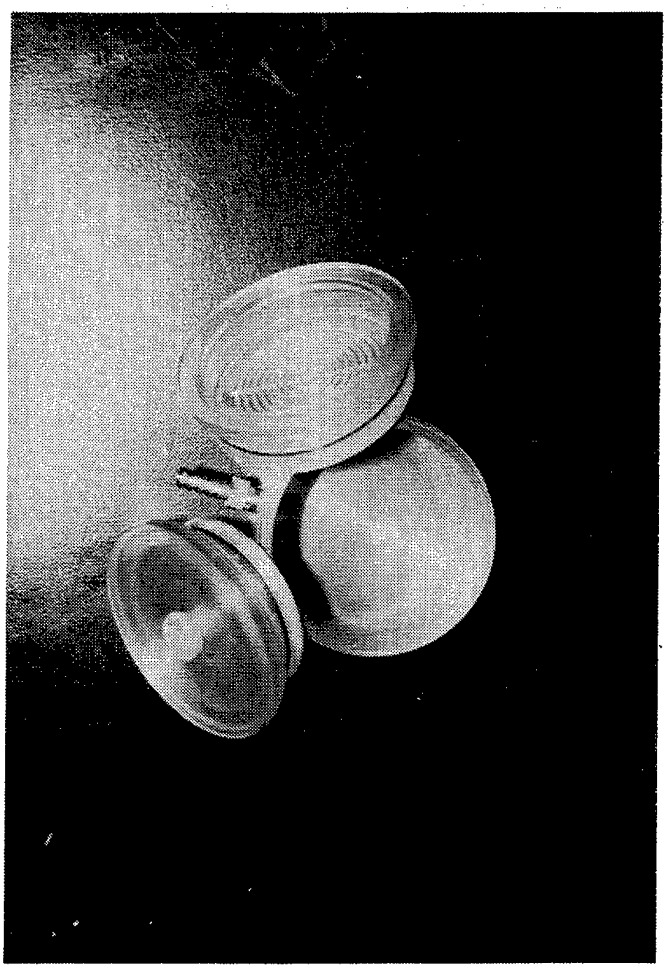

Figure 10.1-3. Vacuum Filtration Unit. This unit has three components and is autoclavable.

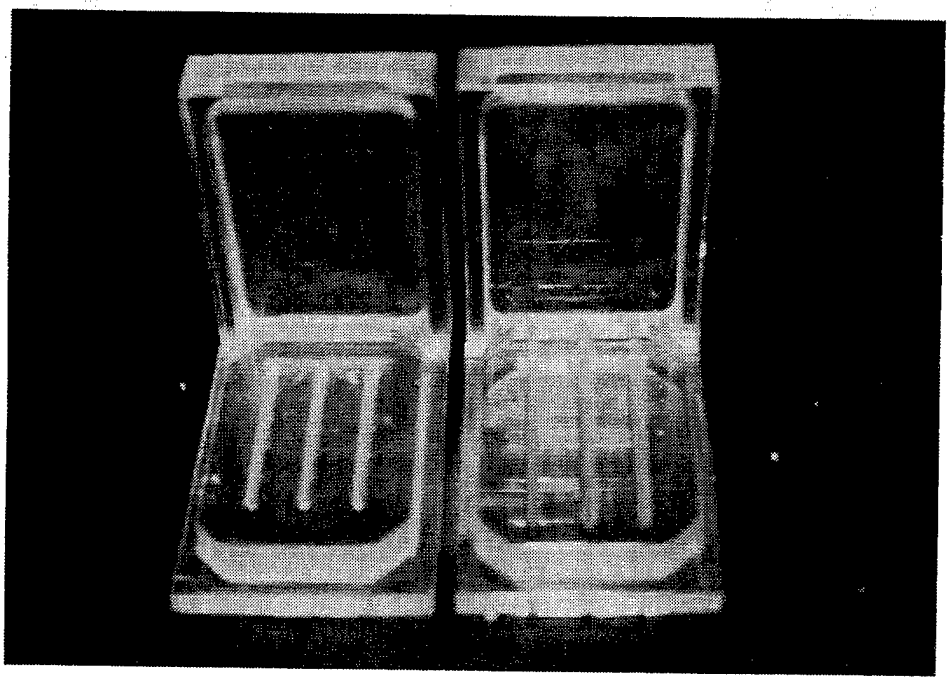

Figure 10.1-4. Small Volume Reagent Reservoir.

Combination Rotorod and Running Wheel Training Apparatus

The THRU Research Engineering Group was tasked to fabricate and design a second rotorod/treadmill apparatus according to the method in Brewer et al., 1992. The rotorod and running wheel will be independent of an inhalation chamber for use in training and conditioning animals (Figure 10.1-5). The training equipment did not require any housing, but is simply a free-standing replica of previous special test equipment whose design and development was done in conjunction with the Navy personnel intending to use it.

The training wheel was designed to enhance the rodents' performance in the exposure apparatus due to the increased training time afforded the animals. A variable speed motor and feces tray were added. The rotorod was balanced for exact stopping and has one door on the outside circumference.

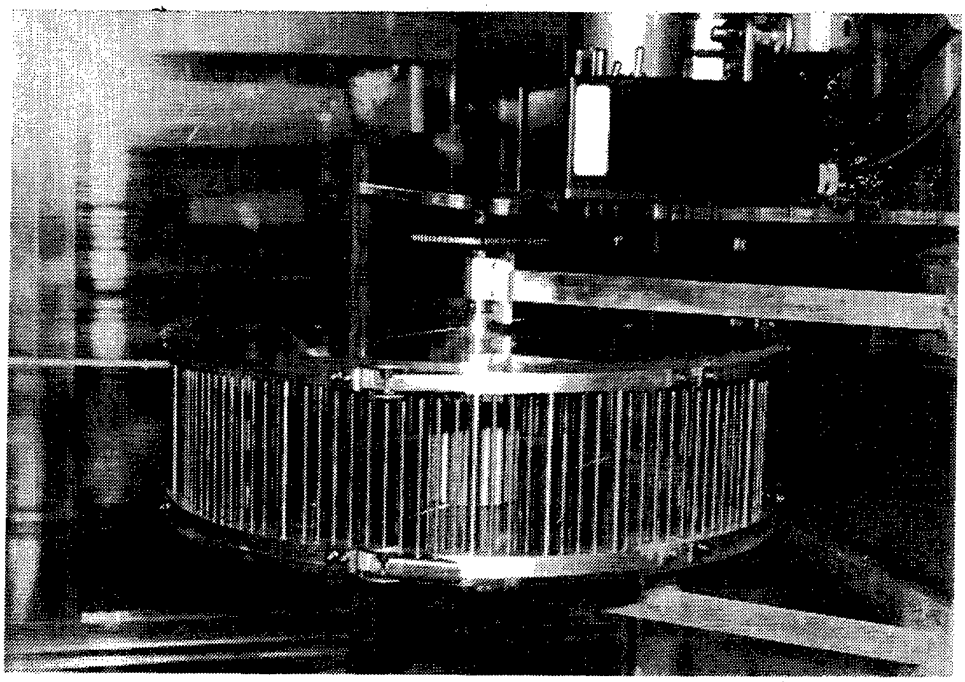

Figure 10.1-5. Combination Rotorod and Running Wheel Training Appartus. This rotorod/running wheel was designed to function independent of inhalation chambers.

Gas-Uptake System

The Air Force Medical Research Unit requested the design and fabrication of a gas-uptake system. The system included the construction of racks, panels, and lids (Figure 10.1-6). The lids were made of stainless steel and consisted of a gas flow line, return line, oxygen sensor, humidity probe, and a thermocouple.

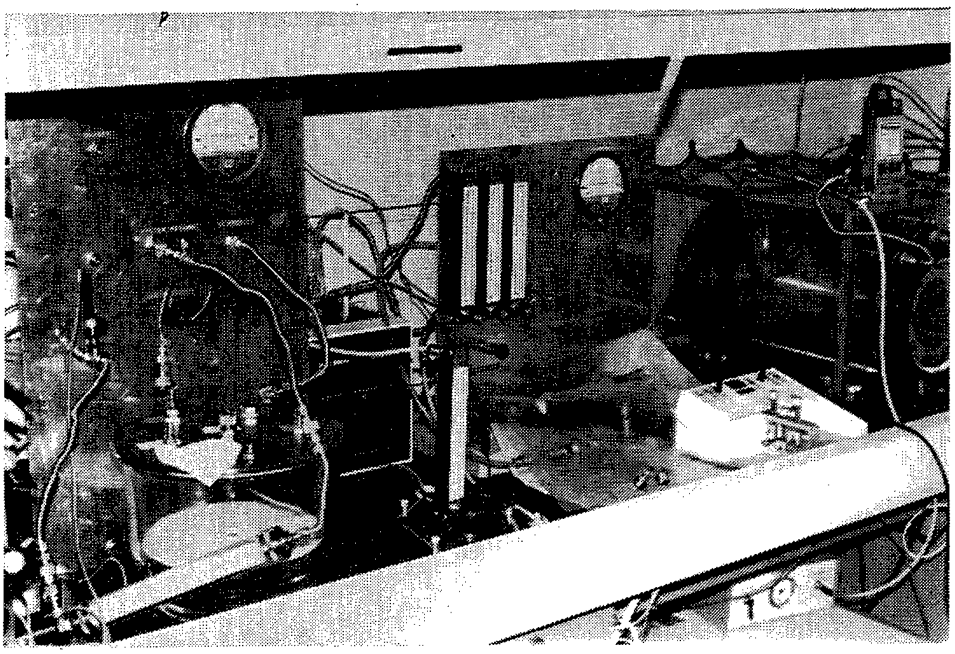

Figure 10.1-6. Gas-Uptake System. Stainless steel lid and exposure system with ancillary connections is shown on left.

The panel to control the system had several functions. Gas was pumped to two transducers connected to a control line that was checked by the humidity probe and housing of Neotronics X90 controls with magnehelic and electrical controls. The racks were 12 in. wide, 18 in. long, and 30 in. high with five shelves. The plexiglas racks held all of the electronic equipment including the dew-point hygrometer, the integrator, etc. This system is compact to fit in an air control hood for safety. Two complete systems were fabricated.

Autosampler Thermostatically Controlled Chambers

The Armstrong Laboratory Comparative Medicine Branch requested the design and fabrication of a thermostatically controlled chamber (7 in. × 17 in. × 19.5 in.) for use with a Tekmar Autosampler so that investigators could incubate 100 samples simultaneously. The incubation system constantly circulates air to ensure uniform temperature throughout the chamber (Figure 10.1-7). The system was designed to be programmed by a digital temperature controller, ranging from 20 to 50 °C. The air is circulated by two fans with individual heaters in a temperature control box sitting behind the autosampler. The thermocouple was a "T" type (with an accuracy of 0.2 °C) located in the middle of the chamber. The chamber consisted of Lexan plastic with a Buna rubber basket around the bottom for air tightness. A handle was added for easy lifting.

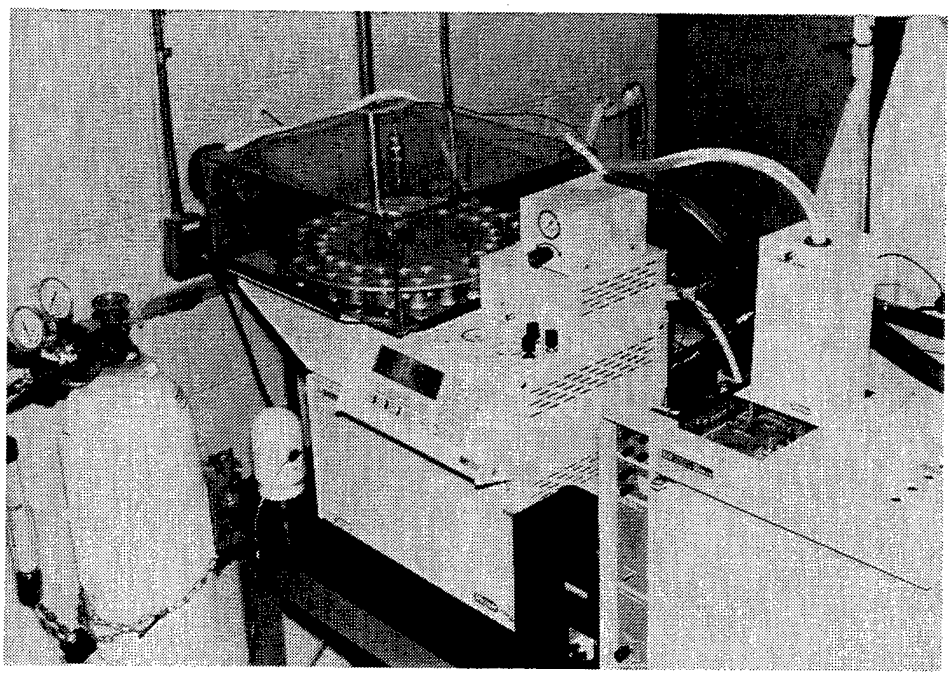

Figure 10.1-7. Autosampler Thermostatically Controlled Chamber.

Nose-Only Chamber Exposure Apparatus

The Navy Medical Research Unit requested the modification/design of a wire-mesh restraint to coordinate with a THRU exposure chamber. The purpose of this modification was to convert the chamber to a nose-only exposure apparatus without rebuilding the entire system. This modification required the temporary replacement of one of the chamber's side windows with a plexiglas window containing nose-only exposure ports. A 3.5-in. diameter plexiglas chamber window collar with two inner "O-rings" was adhered to the window to provide an air-tight fit (Figure 10.1-8).

The stainless steel wire mesh restraints have a unique nose-only apparatus attachment. This apparatus exists as two pieces. The inner piece is designed with finger grips, tapers, and three "O-rings" (two for sealing and a third for holding dental dam). No restraints were needed around the dental dam and the neck of the rats because the restraint's design secures the rat's feet, keeping the rat's head in place. The second piece is the locking piece for the rat's feet, the mesh, and the sealant. The study request stipulated that the chamber have six ports and eight nose pieces. The chamber will be used for determining the toxicity of SFE (solid fire extinguishant) (a powder fire extinguishant proposed as a Halon replacement). SFE is a dry powder agent in tablet form that, when ignited, produces a fine aerosol of potassium salts that extinguishes the fire. In order to conduct a series of aerosol characterization studies and subsequent inhalation toxicity studies, the THRU was requested to fabricate an ignitor/aerosol generation assembly that could be readily adapted for use with whole-body inhalation exposure chambers. This device was fabricated out of 3-in. stainless steel pipe with access ports for thermocouples and a Ni-chrome wire ignitor.

Rodent Ambient Temperature Box and Restrainers

In response to a request from AL/OET, a 12-place ambient temperature isolation box and 24 rodent restrainers were designed and fabricated to be used as a non-invasive tail-cuff method for determining the cardiovascular effects of xenobiotics. The objective of the project was to design a system to minimize the data variability caused by fluctuations in ambient temperature during tail-cuff monitoring.

The isolation box has a programmable controller to control and adjust the temperature for accurate settings ranging from 20° to 37 °C (Figure 10.1-9). Constant fresh air circulates through concentric holes in the top level of four double shelves. The bottom of the box is used as an excreta tray. The double walls feature built-in shutters for blowing air through the adjustable louvers on each side of every shelf, creating air movement similar to laminar flow. The box is 16.5 in. × 24 in. × 27 in. with holes in the back for connecting test equipment wires, and there are sliding doors in the front for easy access.

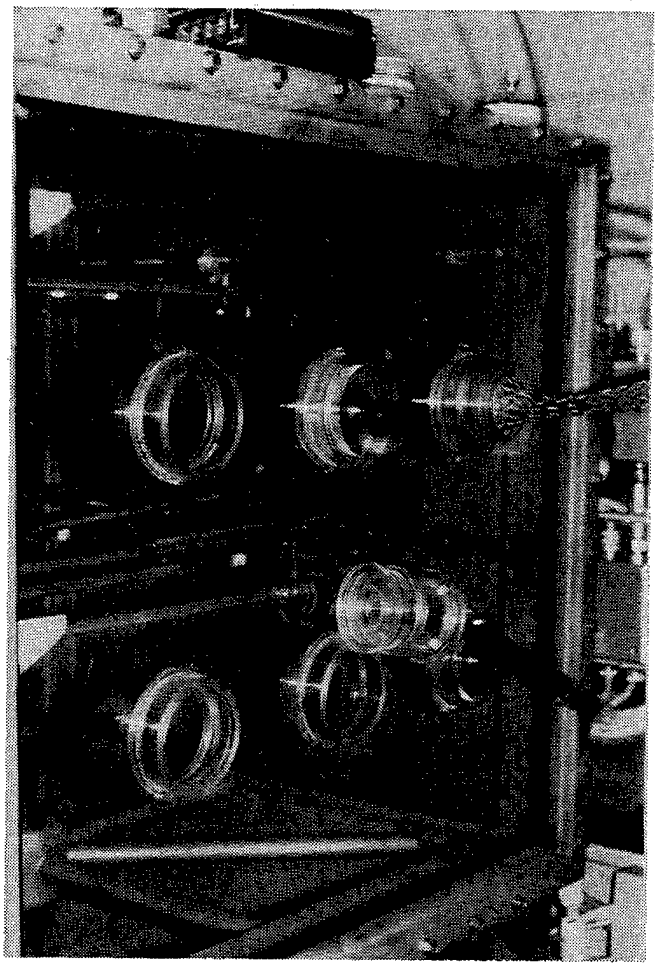

Figure 10.1-8. Nose-Only Chamber Exposure Apparatus. Animal Restraints are shown connected to the top and bottom right ports.

Two sizes of restrainers were fabricated. The first was a 2.5 in. (inside diameter) by 8.5 in. (length) restrainer to fit 200 to 400 g rats; the second was a 3 in. by 10 in. restrainer to fit 400 to 600 g rats. Reducing inserts were constructed for both restrainers for better animal fitting. All holders were made of clear plexiglas and provided mounting arrangements for the IITC tail-cuff sensors. The restrainers contain adjustable sizers with tail sections that are held by snaps. The restrainers allow the animal to walk forward into the restrainer instead of being strapped backwards in their holders. The holders have grooves along the bottom to keep the animals and the restrainers clean of excreta.

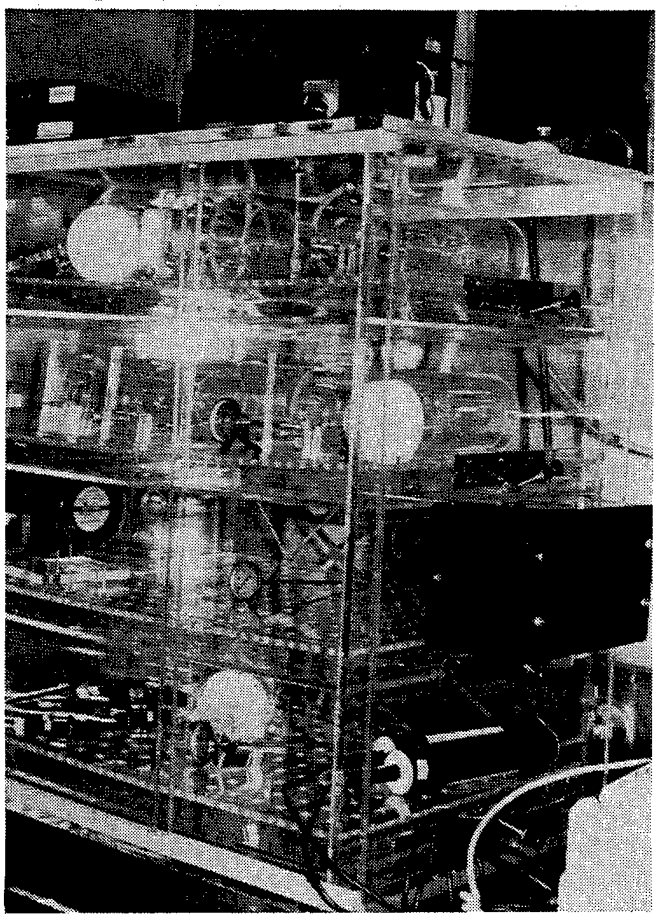

Figure 10.1-9. Rodent Ambient Temperature Box and Restrainers.

REFERENCES

Brewer, J.A., W.B. Sonntag, and E.C. Kimmel. 1992. Combination Rotorod/Treadmill for Rodents. In: 1991 Toxic Hazards Research Unit Annual Report. (H. Wall, D. Dodd, A. Vinegar, H. Clewell eds.) AL-TR-1992-0142, NMRI 92-97, Wright Patterson AF Base, Ohio. p 271.

Cannon, W.C., E.F. Blanton, and K.E. McDonald. 1983. The flow-past chamber: An improved nose-only exposure system for rodents. *Am. Ind. Hyg. Assoc. J.* 44: 923–928.

10.2 MATHEMATICS AND STATISTICS

C.D.Flemming

The THRU's Biometry staff was actively involved in the support of several research projects such as the species differences in skin penetration study, a review of risk analysis procedures for trichloroethylene using the Monte Carlo method coupled with physiologically based pharmacokinetic models, the JP-8 urine analysis study, and the Naval subpopulation statistical study. Important papers reviewed by the Biometry staff included a reproductive toxicology paper presented at the last Tri-Service Toxicology Conference and a paper about a novel model-based approach to the mathematical analysis of dose–response curves. Biometry personnel also assisted with the development of a method for non-cancer risk analysis.

The THRU's statistician assisted various Tri-Service Toxicology personnel in the use of RS/1, SAS, BMDP, and other software packages and aided several investigators with the development of protocols. Power analysis was used on several protocols. The statistician was involved with the incorporation of mathematics and statistics in research plans that will also be applied to new research projects. Programming in FORTRAN, SAS, BMDP, and RPL was performed when required. Informal training in mathematics, statistics, programming and computers was provided to individual Tri-Service Toxicology personnel.

10.3 PATHOLOGY SUPPORT (NECROPSY AND HISTOLOGY)

J.R. Latendresse

Necropsy support was provided in accordance with protocol requirements, standard operating procedures, or as determined by the veterinary pathologists. Routine, accepted methods of anesthesia were followed for terminal bleeding or euthanasia of laboratory animals. Necropsy procedures included determination of terminal body weights, detailed dissection, weighing of required organs, and collections and fixation of gross lesions and other required tissues for light microscopic examination.

Histologic processing of tissues included trimming, orientation of tissues in embedding cassettes, paraffin embedding, microtome sectioning of tissues to specified thickness, and applications for routine or special staining and coverslipping. uniformly processed, high quality slides were prepared for reviewing by veterinary pathologists. A pathology specimen archive is maintained with controlled access as required by Good Laboratory Practices.

The histology/necropsy support technicians have acquired and implemented new skills in tissue processing required for molecular pathology methods now being used extensively in the laboratory. These new tissue processing requirements include unique fixation protocols, sterile sectioning procedures, and microwave processing of tissue to enhance antigen, DNA and RNA retrieval. The tissue staining methods are also unique. The stains are largely performed on a state-of-the-art, computer-based automated stainer operated by histology personnel.

Necropsy personnel played a significant role in the assessment of a computer-based Toxicology Data Management System that is being considered as a potential replacement for the current system. Necropsy personnel worked in concert with the pathologists to evaluate two of the interactive modules (necropsy and histopathology). The tentative replacement program would be able to be operated from a network platform, eliminating the VAX as an operational requirement for the management of toxicology data.

Pathology support was recently expanded by the addition of an electron microscopy technologist to the Toxic Hazards Research Unit (THRU) staff. This has enhanced our ultrastructural-assessment capability and function utilizing both transmission and scanning electron microscopy (EM). In less than six months, the EM laboratory has become fully operational and is already being utilized for two THRU studies (F35 & A05).

Necropsy/histology staff members processed the following research animals during the past reporting period (Tables 10.3.1 and 10.3.2).

TABLE 10.3-1. TOTAL ANIMALS LISTED BY SPECIES AND RESULTING NUMBER OF SLIDES

Species	Animals	Slides
Rats	1461	7787
Mice	602	9858
Pig	3	3
Pigeon	2	46
Monkey	7	55
Dog	5	18
G. Pig	21	12
Frog	2	18
Rabbit	1	6
Human	3	35
Hamster	6	6
Gerbil	4	4

TABLE 10.3-2. NUMBER OF ANIMALS PROCESSED BY MONTH AND RESULTING NUMBER OF SLIDES

Date	Number of Animals (in Species)	Number of Slides (in Type)
October 1993	269 Rats	971 Rat
	6 Monkeys	—
	—	96 Mouse
	—	6 G. Pig
November 1993	83 Rats	1539 Rat
	26 Mice	—
	1 Dog	—
	—	—
December 1993	53 Rats	903 Rat
	4 Mice	13 Mouse
January 1994	327 Rats	145 Rat
	23 Mice	18 Mouse
	2 G. Pigs	6 G. Pig
	1 Frog	12 Frog
	1 Human	—
February 1994	263 Rats	954 Rat
	16 Mice	9 Mouse
	18 G. Pigs	—
	1 Rabbit	6 Rabbit
	—	10 Human
March 1994	178 Rats	1070 Rat
	21 Mice	15 Mouse
	1 G. Pig	—
	—	6 Hamster
April 1994	30 Rats	708 Rat
	17 Mice	375 Mouse
	—	1 Dog
May 1994	84 Rats	1240 Rat
	21 Mice	30 Mouse
	3 Pigs	3 Pig
	1 Pigeon	1 Pigeon
	1 Monkey	1 Monkey
	3 Dogs	3 Dog
	2 Humans	—
June 1994	16 Rats	93 Rat
	19 Mice	29 Mouse
	1 Frog	6 Frog
	—	25 Human
	—	5 Dog
July 1994	21 Rats	55 Rat
	260 Mice	1104 Mouse
	—	4 Gerbil
August 1994	23 Rats	55 Rat
	189 Mice	4004 Mouse
	1 Pigeon	25 Pigeon
	1 Dog	4 Dog
September 1994	114 Rats	53 Rat
	6 Mice	4048 Mouse

10.4 COMPUTER/ELECTRONICS SUPPORT

J.S. Stokes

The Computer and Electronics Section of Research Support provided support for the Armstrong Laboratory/Toxicology Division (AL/OET) VAX minicomputer, and for personal computer (PC), data acquisition and telecommunications systems hardware and software utilized by Toxic Hazards Research Unit (THRU) and Toxicology Division personnel. The following itemization illustrates a few of the services provided during this reporting period by Computer and Electronics Support.

- Wrote a dBASE III program to assist the AL/OET Hazardous Waste Coordinator with hazardous waste turn-in.
- Instructed the THRU's Property Management Custodian in the use of the property management program, which was written in dBASE III.
- Provided telephone and computer support services in the relocation of THRU personnel and computer systems, as follows.

Building 29:	To:
S. Godfrey	Building 429, Room 3B
M. Schneider	Building 79, Room 154E
AutoCAD System	Building 79T, Room 6
Building 79:	
H. Barton	Room 147B to Room 154C
W. Brashear	Room 147F to Room 125
D. Dodd	Room 154F to Room 154D
P. Fleenor	Room 154C to Room 154F
Building 79B:	
D. Courson	Building 79T, Room 2
W. Malcomb	Building 79T, Room 4
Building 79T:	
M. Ketcha	Building 79, Room 185
D. Mahle	Building 79, Room 185
W. Malcomb	Room 4 to Room 5
G. Randall	Building 79, Room 126A
S. Salins	Building 79, Room 150
R. Wolfe	Building 79, Room 149
Building 429:	
C. Flemming	Building 79, Room 147D
ToxInfo System	Building 79, Room 147C

- Maintained stock of PC supply items for THRU computer users.
- Attended the PathTox Users Meeting in April. Presentations ranged from new PathTox capabilities to areas such as software validation.
- Extended the THRU's Lantastic local area network (LAN) printer sharing system, which provides laser and dot matrix printing capabilities for all THRU front office personnel, to include Room 154D.
- Provided support and training for the THRU's Precision Measurement Equipment Laboratory custodian.
- Provided audio/visual and computer support for the 1993 Annual Toxicology Conference held at the Hope Conference Center (Study No. C04), as well as for the recent Trichloroethylene Workshop held at the Fairborn, Ohio, Holiday Inn Conference Center (Study No. F32).
- Worked with AL/OET Computer Support Personnel in planning and installing the Pathworks LAN in Buildings 79 and 824 for the Army. Installation of the LAN is continuing in Buildings 79, 838, and 824.
- Provided system management for Digital Equipment Corp. VAX computer in Building 79. Also provided support for users of Building 79 VAX computer.
- Wrote a VAX program (TEAMS) to allow Tri-Service E-mail to be sent to several functional groups of VAX users.
- Added capabilities of pH monitoring and control to rat liver perfusion data acquisition system/hardware and software previously constructed for LCDR John Wyman at NMRI/TD in support of Study No. N10.

10.5 QUALITY ASSURANCE

M. G. Schneider

The Quality Assurance Coordinator (QAC) presented a poster, "Building an Effective Training Program," at the Society of Quality Assurance Annual meeting held in San Francisco, CA. This material was published in the journal, "Quality Assurance: Good Practice, Regulation, and Law," as part of the meeting proceedings. Training in total Quality Assurance and training methodology was received at this meeting. The QAC participated in the activities of the Mid West Regional Chapter of the Society of Quality Assurance as chair of the By-Laws Committee and attendance at two regional meetings. Training covering computer validation policies, sponsor/subcontractor relationships, personal leadership, and clinical Good Laboratory Practices (GLP) was received at these meetings.

The Quality Assurance Unit (QAU) hosted a seminar and visit by Mrs. Patricia O'Brien Pomerleau, Quality Assurance Manager for the Chemical Industry Institute of Toxicology. The subject of her seminar and one-on-one discussions with Tri-Service Toxicology staff was Quality Assurance for Life Science research. All staff involved in the Tri-Service Toxicology research effort were invited to participate. Follow-up with attendees indicated the information presented was very useful and that the approach to Quality Assurance described could serve as a basis for a Tri-Service Toxicology QA program.

The QAU staff attended specialized training on the software installed on the new computers received during the past year, a one-day course in technical writing, and a one-day seminar on self-directed work teams. The QAC also attended a one-day course on project management. The QAC conducted orientation training in the quality assurance program for all new ManTech staff. The QA Associate (QAA) attended training for GLP compliant archiving. The QAA participated on the program management team during the Conference on Temporal Aspects in Risk Assessment for Noncancer Endpoints and the 1994 HazMat/Pollution Prevention Conference.

The QAC was relocated to office space located in Room 3, Building 429. Systems office furniture was installed to permit housing of three staff in this space.

The QAC conducted protocol reviews, and procedure, data, and final report audits for Study Requests A02(TNB), A03(LP1846), A04(TNB), A08(Unicharge), F04((JP-4), F11(Mixtures), F22(ADN), F23(TCA), F25(CF_3I), F26(Toluene), F29(ADN), F29A(ADN), F35(TCE), and F37(CF_3I).

The QAC acted as ManTech study coordinator for A01(QA program), A04(QA Program and fetal evaluation subcontract), and F27(Building 79 research quality water system).

The QAA completed technical editing for one letter report for the Toluene Study Request F26. Six manuscripts for peer-reviewed publication were edited for Study Requests F04(JP-4), F08(Halon

Replacements), F11(Mixtures), F15(Lactational Transfer), and F23(TCA). Nine technical reports were edited by the QAA for Study Requests A02(TNB), A03(LP1846), F12(Partition coefficients), F20(QSAR Models), F21(Halon Replacements), F22(ADN), F25(CF_3I), F28(TPH), and N12(Hydrazine).

Ten SOPs were reviewed, prepared for approval, distributed, and placed in the SOP Manuals located in laboratory work areas by the Quality Assurance staff. Equipment to reestablish the study records archive was purchased and installed in Room 3D, Building 429. The useable space in this archive was effectively doubled, and the backlog of filing study records can be completed. The pathology slides archive was relocated from an unsecured area of Building 79 to Room 3D, Building 429. Space was provided to allow for an approximate 20% growth of the slide archive. Thirty-two thousand records were sorted and catalogued for microfilming. Duplicate rolls of microfilm were produced and stored at separate locations as has been standard procedure. The records were then filed in the records archive.

10.6 HEALTH AND SAFETY

M. G. Schenider

The ManTech Health and Safety Representative (HSR) worked with the Technical Area Group leaders and the Facilities Space Committee in the development of a Room Monitor Program. The intent of this program was to identify an individual with responsibility for activity, equipment, and supplies in each of the Armstrong Laboratory/Toxicology Division (AL/OET) work spaces. The HSR developed and coordinated the initial training for the thirty-plus individuals named as room monitors. Room monitors were drawn from the Department of Defense (DoD) military and civilian, as well as contractor staff.

A Tri-Service Toxicology bloodborne pathogen exposure control plan was developed by the HSR using various existing documents as a basis. The HSR attended local safety seminars where training and updates on occupational health and safety regulations were received.

The four Thomas Domes located in Room 151, Building 79, were removed during the past year. The HSR monitored the planning and operational process to assure staff safety and to minimize disruption of normal laboratory operations.

Mr. W.J. Malcomb, Research Engineering, provided instruction in CPR and first aid to twelve staff members. Mr. Malcomb is certified by the American Red Cross. ManTech staff who work in laboratory areas received training in Wright-Patterson Air Force Base (WPAFB) hazardous waste management policy and procedures, Occupational Safety and Health Administration's (OSHA's) laboratory chemical standard, and the OSHA bloodborne pathogen standard. ManTech staff who were designated as hazardous waste satellite point managers received specialized training from 2Lt W. Hurtle, AL/OET Hazardous Waste Coordinator. Formal documentation of safety training was implemented using a format which complements Air Force policy. The HSR provided safety orientation training for all new ManTech staff.

The process of integrating the AL/OET hazardous material program into the WPAFB HazMat Cell continued at a cautious pace. The HSR continued to maintain the chemical inventory for AL/OET. The chemical tracking system which has been in place for five years was continued. The process of obtaining material safety data sheets for all chemicals present in the laboratory continued. The HSR provided WPAFB Environmental Management with data for tracking use of materials identified in the EPA-17 and the Ozone Layer Depleting Chemicals listings.

ManTech staff were provided with appropriate safety equipment as required by specific Work Assignments. Purchase agreements were established with local vendors for safety glasses, safety shoes, and labwear. During the past year the labwear agreement was canceled, and items such as labcoats and scrubs were purchased by the Air Force for ManTech use. Laundry of these items was brought into the lab using equipment purchased and maintained by AL/OET.

The medical surveillance program was continued. This program included pre-employment, annual, and exit physicals. The vendor provided emergency care for work related injuries, follow-up examinations for work release, consultation for pregnant staff or suspected work related illness, and initiation of Workers Compensation paperwork. Hepatitis B immunization was offered to staff who might encounter bloodborne pathogens in their jobs. The HSR arranged for the reading of tuberculin test results by ManTech staff, thereby saving the time and expense of employees traveling back to the physical site. All ManTech staff were offered annual physicals.

The formal THRU safety committee ceased to function during the past year. A critical point had been reached where greatly expanded membership was needed to continue committee practices such as the monthly safety inspections of areas where ManTech employees worked. A Tri-Service Safety Council that will address the overall safety program is in the stages of development. Issues and requirements specific to ManTech will be handled by the HSR. The committee hosted a safety appreciation lunch for all ManTech staff to recognize the total organizational safety program results.

Work areas occupied by ManTech staff were subject to quarterly safety inspections by Air Force safety personnel from Brooks AFB, WPAFB, and Armstrong Laboratory North. Specific programs as Environmental Compliance and Management Program, Radiation Safety, Hazardous Waste (chemical and biological), and Explosives Safety were also inspected by the Air Force. The HSR accompanied these inspection teams. Response by ManTech to the findings was always timely. The HSR also conducted spot and monthly safety inspections. Issues of immediate concern were addressed with staff and supervisors on-the-spot.

APPENDIX A

TOXIC HAZARDS RESEARCH UNIT PERSONNEL
AS OF 30 SEPTEMBER 1994

Darol E. Dodd, Ph.D., DABT
LABORATORY DIRECTOR

RESEARCH SUPPORT
Patricia M. Smith
Supervisor

Staff:
Angell, Mary Ann
Brooks, Shelia D.
Stokes, James

QUALITY ASSURANCE/SAFETY
Mathias G. Schneider, Jr.
Coordinator

Staff:
Godfrey, Susan M.

ADMINISTRATION
Lois A. Doncaster
Supervisor

RISK ASSESSMENT
Hugh A. Barton, Ph.D.
Supervisor

Staff:
Flemming, Carlyle D.

TOXICOLOGY
Darol E. Dodd, Ph.D., DABT
Manager

Staff:
Abernathy, Frank W. Ph.D.
Courson, David L.
Drerup, Joanne M.
Freedman, Marcia L.
Godfrey, Richard J.
Kinkead, Edwin R.
Lane, John W.
Latendresse, John R., D.V.M., Ph.D., DACVP
Leahy, Harold F.
Malcomb, Willie J.
Neely, Gloria A.
Nicholson, Jerry W.
Parish, Margaret A.
Randall, Gia M.
Salins, Stephanie A.
Sonntag, William B.
Wolfe, Robin E.

BIOLOGICAL SIMULATION
Allen Vinegar, Ph.D.
Manager

Staff:
Brashear, Wayne T., Ph.D.
Buttler, Gerry W.
Byczkowski, Janusz Z., Ph.D., D.Sc.
Collins, Richard, Ph.D.
Frazier, John M., Ph.D.
Ketcha, Marcia M.
Kuhlmann, Karl J.
Mahle, Deirdre A.
Pollard, Daniel L.
Seckel, Constance S.

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Seckel, Constance S.

APPENDIX B

PRODUCTS LIST FOR 1994[1]

AWARDS AND HONORS

- **Best Research Paper for 1993 presented by the Society of Toxicology Inhalation Specialty Section, 14 March:**

  Gearhart, J.M., C.S. Seckel, and A. Vinegar. *In vivo* metabolism of chloroform in B6C3F1 mice determined by the method of gas uptake: The effects of body temperature on tissue partition coefficients and metabolism, *Toxicology and Applied Pharmacology* **119**: 258–266, 1993.

- **Best Research Poster (Research and Development) presented at the Thirty–Fifth Navy Occupational Health and Preventive Medicine Workshop, 1 March, and Best Manuscript Award presented by *Phi Zeta* (Veterinary Medical Honor Fraternity) in collaboration with The Ohio State University:**

  Latendresse, J.R., C.C. Capen, and C.L. Brooks. Pathogenesis of cholesteryl lipidosis of ovarian interstitial and adrenocortical cells in F-344 rats caused by triaryl phosphates.

- **Best Poster presented by the TriService Toxicology Division, 10 March:**

  Kinkead, E.R., R.E. Wolfe, S.A. Salins, C.R. Miller, and **J.R. Latendresse.** Single-generation reproduction study of 1,3,5–trinitrobenzene in the diet of Sprague–Dawley rats.

- **Best Poster (2nd Place) presented by the TriService Toxicology Division, 10 March:**

  Williams, R.J., **A. Vinegar,** J.W. Fisher, A.M. Jarabek, and J.N. McDougal. Species extrapolation of 2,2-dichloro-1,1,1-trifluoroethane kinetics: A corollary approach to physiologically based pharmacokinetic modeling.

- **Certificate of Appreciation presented by the Sponsors (DoD, EPA, ATSDR) of the Toxicology Conference, 20 April, to L. Doncaster** for 30 years of dedication and service in coordinating the annual toxicology conferences for the DoD.

- **Certificate of Achievement presented by the Defense Logistics Agency (DCMAO-Atlanta), 21 February, to M. Angell and ManTech Environmental** for meeting and/or exceeding small disadvantaged business goals for fiscal year 1993.

[1] Current or previous employees are identified in bold type.

INVITED PRESENTATIONS

- **Barton, H.A., at the Trichloroethylene Science Workshop, Williamsburg, 2–3 December, 1993:**

  Animal evidence for the carcinogenicity of trichloroethylene.

- **Byczkowski, J.Z., at the annual meeting of the Drug Information Association, Washington, D.C., 6–7 June:**

  Linked PBPK model and cancer risk assessment for breast-fed infants.

- **Frazier, J.M., at "INVITRO 94", 8th International Workshop on *In Vitro* Toxicology, Kartause Ittingen, Switzerland, 20–23 September:**

  Application of *in vitro* systems to the predictions of *in vivo* biokinetics.

- **Frazier, J.M., keynote speaker at the European Center for the Validation of Alternative Methods Symposium (opening), Ispra, Italy, 17–18 October :**

  Interdisciplinary approach to toxicity test development and validation.

- **Latendresse, J.R., at the Lilly Pharmaceutical Research Laboratories, Greenfield, 5 July:**

  Pathogenesis of cholesteryl lipidosis in adrenocortical and ovarian interstitial cells in F-344 rats caused by triaryl phosphates.

JOURNAL PUBLICATIONS

Andersen, M.E., **H.J. Clewell III, D.A. Mahle, and J.M. Gearhart.** Gas uptake studies of deuterium isotope effects on dichloromethane metabolism in female B6C3F1 mice *in vivo*, *Toxicology and Applied Pharmacology* **128:** 158–165, 1994.

Barton, H.A., J.R. Creech, **C.S. Godin, G.M. Randall, and C.S. Seckel.** Chloroethylene mixtures: Pharmacokinetic modeling and *in vitro* metabolism of vinylchloride, trichloroethylene, and *trans*-1,2-dichloroethylene in rats, *Toxicology and Applied Pharmacology*, in press.

Barton, H.A., D.R. Mattie, and W.B. Peirano. Introduction [to the Conference on the Risk Assessment Paradigm After Ten Years], *Risk Analysis* **14:** 217, 1994.

Barton, H.A., D.R. Mattie, and W.B. Peirano (eds.). Special Issue: The Risk Assessment Paradigm After Ten Years, *Risk Analysis* **14:** 217–378, 1994.

Byczkowski, J.Z. Linked PBPK model and cancer risk assessment for breast-fed infants, *Drug Information Journal*, in press.

Byczkowski, J.Z. and J.W. Fisher. Lactational transfer of tetrachloroethylene in rats, *Risk Analysis* **14:** 339–349, 1994.

Byczkowski, J.Z. and J.W. Fisher. A computer program linking physiologically based pharmacokinetic model with cancer risk assessment for breast-fed infants, *Computer Methods and Programs in Biomedicine*, in press.

Byczkowski, J.Z., J.M. Gearhart, and J.W. Fisher. Occupational exposure of infants to toxic chemicals via breast milk, *Nutrition* **10:** 43–48, 1994.

Byczkowski, J.Z., E.R. Kinkead, H.F. Leahy, G.M. Randall, and J.W. Fisher. Computer simulation of the lactational transfer of tetrachloroethylene in rats using a physiologically based model, *Toxicology and Applied Pharmacology* **125:** 228–236, 1994.

Godin, C.S., J. He, J.M. Drerup, and J.F. Wyman. Assessment of biomarkers of vascular toxicity associated with exposure to propylene glycol-1,2-dinitrate, *Toxicology Letters*, in press.

Goeke-Flora, C.M., **J.F. Wyman,** B.M. Jarnot, and N.V. Reo. Effect of the peroxisome proliferator perfluoro-n-decanoic acid on glucose transport in the isolated perfused rat liver, *Chemical Research Toxicology*, in press.

Jarabek, A.M., J.W. Fisher, R. Rubenstein, J.C. Lipscomb, R.J. Williams, **A. Vinegar,** and J.N. McDougal. Mechanistic insights aid the research for CFC substitutes: Risk assessment of HCFC-123 as an example, *Risk Analysis* **14:** 231–250, 1994.

Jepson, G.W., D.K. Hoover, R.K. Black, J.D. McCafferty, **D.A. Mahle, and J.M. Gearhart.** A partition coefficient determination method for nonvolatile chemicals in biological tissues, *Fundamental and Applied Toxicology* **22:** 519–524, 1994.

Kinkead, E.R. and R.E. Wolfe. Acute oral and dermal toxicity of quadricyclane, *Acute Toxicity Data*, **6:** 634, 1993.

Kinkead, E.R. and R.E. Wolfe. Acute oral and inhalation toxicity of petroleum-derived JP-4 jet fuel, *Acute Toxicity Data*, **6:** 635, 1993.

Kinkead, E.R. and R.E. Wolfe. Acute oral and dermal toxicity of ammonium dinitramide, *Acute Toxicity Data*, in press.

Kinkead, E.R., R.E. Wolfe, C.D. Flemming, R.A. Soloman, D.R. Mattie, J.H. Grabau, and G.B. Marit. The toxicologic and oncogenic potential of JP-4 vapor: 90-Day continuous inhalation exposure, *Inhalation Toxicology*, in press.

Latendresse, J.R., C.L. Brooks, and C.C. Capen. Pathologic effects of butylated triphenyl phosphate-based hydraulic fluid (BTP) and tricresyl phosphate (TCP) on the adrenal gland, ovary, and testis in the Fischer 344 rat, *Toxicologic Pathology* **22:** 341–352, 1994.

Latendresse, J.R., C.L. Brooks, and C.C. Capen. Toxic effects of butylated triphenyl phosphate-based hydraulic fluid and tricresyl phosphate in female F-344 rats, *Veterinary Pathology*, in press.

Latendresse, J.R., C.L. Brooks, **C.D. Flemming,** and C.C. Capen. Reproductive toxicity of butylated triphenyl phosphate and tricresyl phosphate fluids in F-344 rats, *Fundamental and Applied Toxicology* **22:** 392–399, 1994.

Latendresse, J.R., G.B. Marit, E.H. Vernot, C.C. Haun, and **C.D. Flemming.** Oncogenic potential of inhaled hydrazine in the nose of rats and hamsters after one or ten one-hour exposures, *Fundamental and Applied Toxicology*, in press.

Marit, G.B., M.K. Cooper, and **J.R. Latendresse.** A method for processing and immunohistochemical staining of rat nasal sections, *Journal of Histotechnology*, in press.

Marit, G.B., **D.E. Dodd,** M.E. George, and **A. Vinegar.** Hepatotoxicity in guinea pigs following acute inhalation exposure to 1,1-dichloro-2,2,2-trifluoroethane, *Toxicologic Pathology* **22:** 404–414, 1994.

Rivera, J., **J. Wyman,** D. von Minden, N. Lacy, and D.A. Macys. A facile synthetic procedure for the preparation of highly-pure 2,6-di-tert-butyl-4-nitrophenol (DBNP), *Environmental Toxicology and Chemistry*, in press.

Roth, R.A. and **A.Vinegar.** Action by the lungs on circulating xenobiotic agents, with a case study of physiologically based pharmacokinetic modeling of benzo(a)pyrene disposition, In: Gram, T.E., (ed.), Metabolic activation and toxicity of chemical agents to lung tissue and cells, *International Encyclopedia of Pharmacology and Therapeutics*, Sect. **138:** 89–105, 1993.

Schneider, M.G. Building an effective training program, *Quality Assurance: Good Practice, Regulation, and Law* **3:** 316–318, 1994.

Vinegar, A., R.J. Williams, J.W. Fisher, and J.N. McDougal. Dose-dependent metabolism of 2,2-dichloro-1,1,1-trifluoroethane: A physiologically based pharmacokinetic model in the male Fischer 344 rat, *Toxicology and Applied Pharmacology* **129:** 103–113, 1994.

Wyman, J., J.S. Stokes, M. Goehring,, M. Buring, and T. Moore. Data collection interface for isolated perfused rat liver: Recording oxygen consumption, perfusion pressure and pH, *Toxicology Methods*, in press.

JOURNAL SUBMISSIONS

Cronin, W.J., E.J. Oswajd, M. Shelley, J.W. Fisher, and **C.D. Flemming.** A trichloroethylene risk assessment using a Monte Carlo analysis of parameter uncertainty in conjunction with physiologically based pharmacokinetic modeling, *Risk Analysis.*

Ketcha, M.M., D.A. Warren, C.T. Bishop, **H.A. Barton, and W.T. Brashear.** Analyses of dichloroacetic acid, trichloroacetic acid, trichloroethanol and trichloroethanol glucuronide: Metabolites of trichloroethylene, *Analytical Biochemistry.*

Kinkead, E.R., R.E. Wolfe, C.D. Flemming, D.J. Caldwell, C.R. Miller, and G.B. Marit. Reproductive toxicity screen of 1,3,5-trinitrobenzene administered in the diet of Sprague-Dawley rats, *Toxicology and Industrial Health.*

Kinkead, E.R., R.E. Wolfe, S.A. Salins, C.D. Flemming, H.F. Leahy, D.J. Caldwell, C.R. Miller, and G.B. Marit. Reproductive toxicity screen of liquid propellant XM46 administered in the drinking water of Sprague-Dawley rats, *Toxicology and Industrial Health.*

Lipscomb, J.C., **D.A. Mahle,** C.M. Garrett, and **H.A. Barton.** Dichloroacetic acid: Metabolism in cytosol from mice, rats and humans, *Drug Metabolism and Disposition.*

Rossi, J., M.S. Buring, **W.B. Sonntag,** and G.D. Ritchie. The NMRI/TD roto-wheel: A new apparatus for multiple measures of physical incapacitation, *Behavior Research Methods, Instruments, and Computers.*

Seckel, C.S., H.G. Wall, D.R. Mattie, C.E. Jones, M. Andersen, and **A. Vinegar.** Effects of polychlorotrifluoroethylene (PCTFE) on two Rhesus monkeys following long-term oral dosing, *Journal of Applied Toxicology.*

Wyman, J., R. Fisher, S. Prues, **C. Flemming,** J. Rivera, and K. Brendel. Comparative toxicity of 2,6-di-tert-butyl-4-nitrophenol and other nitrophenols in human and rat hepatic tissue slices, *In Vitro Toxicology.*

TECHNICAL REPORTS

Barton, H.A., D.R. Mattie, and W.B. Peirano. The risk assessment paradigm after ten years: Policy and practice then, now, and in the future, Proceedings of the 1993 Conference on Toxicology, AL/OE-TR-1994-0073.

Barton, H.A., J.Z. Byczkowski, S.R. Channel, B.M. Jarnot, J.C. Lipscomb, and R.J. Williams. Trichloroethylene: Metabolism and other biological determinants of mouse liver tumors, AL/OE-TR-1994-0135.

Brashear, W. and D.A. Reid. QSAR evaluation of Halon 1301 (CF_3Br) and CF_3I, AL/OE-TR-1994-0067.

Collins, R. Improvements in modeling of pulmonary uptake of toxicants, AL/OE-TR-1994-0150.

Creech, J.R., R.K. Black, S.K. Neurath, M.C. Caracci, R.J. Williams, G.W. Jepson, and **A. Vinegar.** Inhalation uptake and metabolism of Halon 1301 replacements, HFC-227ea, HFC-125, and FC-218, Air Force Technical Report (in preparation).

Dodd, D.E. and P.M. Smith eds. 1993 Toxic Hazards Research Unit Annual Report, AL/OE-TR-1994-0119.

Frazier, J.M. Evaluation of *in vitro* alternatives to the dog cardiac sensitization assay, AL/OE-TR-1994-0147.

Godin, C.S., M.M. Ketcha, J.M. Drerup, and A. Vinegar. Metabolism of 2,2-dichloro-1,1,1-trifluoroethane (HCFC-123) by human hepatic microsomes, AL/OE-TR-1994-0149.

Kenny, T.J., C.K. Shepherd, and C.J. Hardy (**D.E. Dodd**, coordinator). Iodotrifluoromethane and iodoheptafluoropropane assessment of cardiac sensitisation potential in dogs, draft report submitted by Huntingdon Research Centre Ltd. to be finalized and submitted as an Air Force Technical Report.

Kinkead, E.R., M.L. Freedman, R.E. Wolfe, and H.F. Leahy. Acute 15-minute, nose-only inhalation exposure of Halon 1301 to male and female Sprague-Dawley rats, AL/OE-TR-1994-0120.

Kinkead, E.R., S.A. Salins, R.E. Wolfe, H.F. Leahy, and J.H. English. Acute toxicity evaluation of halon replacement trifluoroiodomethane (CF_3I), AL/OE-TR-1994-0070.

Kinkead, E.R., S.A. Salins, R.E. Wolfe, and G.B. Marit. Acute and subacute toxicity evaluation of ammonium dinitramide, AL/OE-TR-1994-0071.

Kinkead, E.R., R.E. Wolfe, C.D. Flemming, H.F. Leahy, D.J. Caldwell, C.R. Miller, and G.B. Marit. Reproductive toxicity screen of ammonium dinitramide administered in the drinking water of Sprague-Dawley rats, AL/OE-TR-1994-0162, WRAIR-TR-94-0015.

Kinkead, E.R., R.E. Wolfe, C.D. Flemming, D.J. Caldwell, C.R. Miller, and G.B. Marit. Reproductive toxicity screen of 1,3,5-trinitrobenzene administered in the diet of Sprague-Dawley rats, AL/OE-TR-1994-0144, WRAIR-TR-94-0016.

Kinkead, E.R., R.E. Wolfe, S.A. Salins, C.D. Flemming, D.J. Caldwell, C.R. Miller, and **J.R. Latendresse.** Range-finding study of a reproductive assessment of 1,3,5-trinitrobenzene administered in the diet of Sprague-Dawley rats, AL/OE-TR-1994-0072, WRAIR-TR-94-0006.

Kinkead, E.R., R.E. Wolfe, S.A. Salins, C.D. Flemming, H.F. Leahy, D.J. Caldwell, C.R. Miller, and G.B. Marit. A reproductive toxicity screen of a liquid propellant XM46 administered in the drinking water of Sprague-Dawley rats, AL/OE-TR-1994-0101, WRAIR-TR-94-0008.

Leahy, H.F. Overview of the vapor generation and analysis parameters of the petroleum- and shale-derived fuel studies conducted in Thomas dome exposure chambers at the Toxic Hazards Research Unit Wright-Patterson AFB (Dayton), Ohio 1973-1983, AL/OE-TR-1994-0075.

Mitchell, A.D. (**D.E. Dodd**, coordinator). "Mutagenesis testing of 1,1,1,2,3,3,3-heptafluoropropane (HFC-227ea) using the Ames *Salmonella typhimurium* histitine reversion assay for volatile chemicals, with and without metabolic activation," Final report submitted by Genesys Research, Inc. to be submitted as an Air Force Technical Report.

Mitchell, A.D. (**D.E. Dodd**, coordinator). "*In vivo* mouse bone marrow erythrocyte micronucleus testing of 1,1,1,2,3,3,3-heptafluoropropane (HFC-227ea)," Final report submitted by Genesys Research, Inc. to be submitted as an Air Force Technical Report.

Mitchell, A.D. (**D.E. Dodd**, coordinator). "*In vitro* forward mutation assay of 1,1,1,2,3,3,3-heptafluoropropane (HFC227ea) using the L5178Y/tk$^{+/-}$ mouse lymphoma cell mutagenesis assay (MLA) with colony sizing, with and without metabolic activation," Final report submitted by Genesys Research, Inc. to be submitted as an Air Force Technical Report.

Mitchell, A.D. (**D.E. Dodd**, coordinator). "Mutagenesis testing of iodotrifluoromethane (CF_3I) using the Ames *Salmonella typhimurium* histidine reversion assay for volatile chemicals, with and without metabolic activation," Final report submitted by Genesys Research, Inc. to be submitted as an Air Force Technical Report.

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Mitchell, A.D. (**D.E. Dodd**, coordinator). "*In vitro* forward mutation assay of iodotrifluoromethane (CF_3I) using the L5178Y/tk$^{+/-}$ mouse lymphoma cell mutagenesis assay (MLA) with colony sizing, with and without metabolic activation," Final report submitted by Genesys Research, Inc. to be submitted as an Air Force Technical Report.

Paika, I.J. (**D.E. Dodd**, coordinator). Genetic toxicity evaluation of 1,3,3-trinitroazetidine. Volume I of IV: Results of *Salmonella typhimurium* reverse mutation assay (Ames assay), AL/OE-TR-1994-0069, vol. I.

Paika, I.J. (**D.E. Dodd**, coordinator). Genetic toxicity evaluation of 1,3,3-trinitroazetidine. Volume II of IV: Results of mouse bone marrow micronucleus test, AL/OE-TR-1994-0069, vol. II.

Paika, I.J. (**D.E. Dodd**, coordinator). Genetic toxicity evaluation of 1,3,3-trinitroazetidine. Volume III of IV: Results of gene mutation at the HGPRT locus in cultured chinese hamster ovary cells, AL/OE-TR-1994-0069, vol. III.

Prezioso, J.A., L. Desai, G. FitzGerald, I. Paika, N. DiGiulio, and **D.E. Dodd.** Genetic toxicity evaluation of 1,3,3-trinitroazetidine. Volume IV of IV: Summary report on the genotoxicity of TNAZ, AL/OE-TR-1994-0069, vol. IV.

Williams, R.J., J.R. Creech, R.K. Black, S.K. Neurath, G.W. Jepson, **A. Vinegar, and J.Z. Byczkowski.** Gas uptake kinetics of bromotrifluoromethane (Halon 1301) and its proposed replacement iodotrifluoromethane (CF_3I), AL/OE-TR-1994-0068.

Zhu, S., E. Korytnyski, and S Sharma (**D.E. Dodd**, coordinator). Genotoxicity assays of ammonium dinitramide: I. *Salmonella*/microsome mutagenesis, II. Mouse lymphoma cell mutagenesis, III. *In vivo* mouse bone marrow micronuclei test, AL/OE-TR-1994-0148.

LETTER REPORTS

Dodd, D.E. Genotoxicity of 1,3,3-trinitroazetidine, 31 May.

ABSTRACTS AND PRESENTATIONS

Barton, H.A., C.S. Seckel, G.M. and Randall. Correlating P450 activity *in vivo* and *in vitro* for pharmacokinetic modeling of trichloroethylene, *Toxicologist* **14:** 40, 1994. (Presented at the annual meeting of the Society of Toxicology, Dallas, 13–18 March).

Bel, P., **M.M. Ketcha, D.L. Pollard,** D.J. Caldwell, J.P. Martin, L. Narayanan, and J.W. Fisher. *In vivo* metabolism of 1,3,5-trinitrobenzene in rats. (Presented at the 42nd meeting of the American Society for Mass Spectrometry, Chicago, 29 May–3 June).

Byczkowski, J.Z. and J.W. Fisher. Tetrachloroethylene exposure assessment of breast-fed infants, *Toxicologist* **14:** 45, 1994. (Presented at the annual meeting of the Society of Toxicology, Dallas, 13–18 March).

Byczkowski, J.Z., T. Pravecek, and S. Channel. Computer simulations of lipid peroxidation in precision cut mouse liver slices and experimental calibration of the mathematical model. (Presented at the Miami Valley regional meeting of the Society of Toxicology, Cincinnati, 7 October).

Channel, S.R., T. Pravecek, and **W.T. Brashear.** Metabolism of trichloroethylene in precision-cut human and mouse liver slices, *Toxicologist* **14:** 283, 1994. (Presented at the annual meeting of the Society of Toxicology, Dallas, 13–18 March).

Das, S., **J.Z. Byczkowski,** and J.W. Fisher. Probability analysis of TCE cancer bioassay data in B6C3F1 mice using PBPK/PBPD modeling: A conceptual framework. (Presented at the annual meeting of the Society of Risk Analysis, Baltimore, 4–7 December).

Gearhart, J.M., H.A. Clewell III, and M.E. Andersen. Identification of metabolic parameters in the B6C3F1 mouse for use in a pharmacokinetic risk assessment for methylene chloride, *Toxicologist* **14:** 114, 1994. (Presented at the annual meeting of the Society of Toxicology, Dallas, 13–18 March).

Jarnot, B.M., C.R. Miller, and **M.M. Ketcha.** Pharmacokinetics of TCA and DCA in B6C3F1 mice following drinking water exposure, *Toxicologist* **14:** 116, 1994. (Presented at the annual meeting of the Society of Toxicology, Dallas, 13–18 March).

Kinkead, E.R., R.E. Wolfe, S.A. Salins, J.R. Latendresse, D.J. Caldwell, and C.R. Miller. A reproductive toxicity screen of a liquid propellant (LP) formulation administered in the drinking water of Sprague-Dawley rats. (Presented at the annual meeting of the American College of Toxicology, Williamsburg, 23–26 October).

Kinkead, E.R., R.E. Wolfe, S.A. Salins, J.R. Latendresse, D.J. Caldwell, and C.R. Miller. Single-generation reproduction study of 1,3,5-trinitrobenzene administered in the diet of Sprague-Dawley rats. (Presented at the annual meeting of the American College of Toxicology, Williamsburg, 23–26 October).

Kinkead, E.R., R.E. Wolfe, S.A. Salins, C.R. Miller, and **J.R. Latendresse.** Single-generation reproduction study of 1,3,5-trinitrobenzene in the diet of Sprague-Dawley rats, *Toxicologist* **14:** 161, 1994. (Presented at the annual meeting of the Society of Toxicology, Dallas, 13–18 March).

Kuhlmann, K.J. Smoke production from advanced composite materials. (Presented at a meeting of the American Chemical Society, Washington, D.C., 22–24 August).

Latendresse, J.R., C.C. Capen, and C.L. Brooks. Pathogenesis of cholesteryl lipidosis of ovarian interstitial and adrenocortical cells in F-344 rats caused by triaryl phosphates. (Presented at the Thirty-Fifth Navy Occupational Health and Preventive Medicine Workshop, Virginia Beach, 1 March).

Latendresse, J.R. and G.B. Marit. Oncogenic potential of inhaled hydrazine in rats after a series of one-hour exposures and then held a lifetime. (Presented at the 20th Annual Conference of the American Chemical Society, San Diego, 15–18 March).

Latendresse, J.R., G.B. Marit, and **C.D. Flemming.** Oncogenic potential of inhaled hydrazine in the nose of rats after a series of one-hour weekly exposures. (Presented at the 29th Annual Meeting of the American College of Veterinary Pathologists, Montreal, December).

Lipscomb, J.C., **W.T. Brashear, G.W. Buttler, H.A. Barton, and D.A. Mahle.** *In vitro* metabolism of dichloroacetic acid, a trichloroethylene metabolite, *ISSX Proceedings* **6:** 129, 1994. (ISSN 1061-3439) (Presented at the 6th meeting of the North American International Society for the Study of Xenobiotics, Raleigh, 23–27 October).

Mahle, D.A., W.T. Brashear, and J.C. Lipscomb. Interspecies comparison of trichloroethylene metabolism *in vitro*, *Toxicologist* **14:** 282, 1994. (Presented at the annual meeting of the Society of Toxicology, Dallas, 13–18 March).

Paika, I.J., G.B. Fitzgerald, **D.E. Dodd,** and D.J. Caldwell. Mutagenic evaluation of a highly energetic castable explosive chemical: 1,3,3-Trinitroazetidine (TNAZ). (Presented at the 25th annual meeting of the Environmental Mutagen Society, Portland, 7–12 May).

Tocco, D.R., **S.A. Salins, R.E. Wolfe, and E.R. Kinkead.** Acute and subacute toxicity of ammonium dinitramide. (Presented at JANNAF workshops, Houston, 18–20 April, and San Diego, 1–5 August).

Vinegar, A. Status report on CF_3I toxicity. (Presented at the CF_3I Working Group meeting at the New Mexico Engineering Research Institute, Albuquerque, 22 August).

Vinegar, A. Toxicity summary of Halon 1301 replacement candidates. (Presented at the Halon Replacement Program for Aviation Systems Technology Transition (T2) Team Meeting hosted by the National Institute of Standards and Technology (NIST) at the Gaithersburg Hilton, Gaithersburg, 27–28 October).

Williams, R.J., **A. Vinegar,** J.W. Fisher, A.M. Jarabek, and J.N. McDougal. Species extrapolation of 2,2-dichloro-1,1,1-trifluoroethane kinetics: A corollary approach to physiologically based pharmacokinetic modeling, *Toxicologist* **14:** 40, 1994. (Presented at the annual meeting of the Society of Toxicology, Dallas, 13–18 March).

Wolfe, R.E., S.A. Salins, E.R. Kinkead, D.J. Caldwell, C.R. Miller, and G.B. Marit. A reproductive toxicity screen of a liquid propellant (XM46) formulation administered in the drinking water of Sprague-Dawley rats. (Presented at the annual meeting of the American Industrial Hygiene Conference and Exposition, Anaheim, 24 May).

Yu, K.O., A. Burton, S.R. Channel, J.W. Fisher, **J. Drerup,** and D. Tillitt. Carrier effects of dosing the H4II cells with PCB (3,3',4,4'-tetrachlorobiphenyl) in DMSO or isooctane. (Presented at the meeting of the Society of Environmental Toxicology and Chemistry, Denver, 31 October–2 November).

COLLABORATIVE JOURNAL PUBLICATIONS

Abernathy, F. and E. Pacht. Alteration of ATP and other cellular nucleotides following sublethal oxidant injury to the rat type II alveolar epithelial cells. *American Journal of Medical Science,* in press.

Abernathy, F. and E. Pacht. Prevention of intracellular ATP depletion following sublethal oxidant injury to the type II alveolar epithelial cells with exogenous glutathione and n-acetyl cysteine. *American Journal of Medical Science,* submitted.

Barton, H.A. and M.A. Marletta. Comparison of aniline hydroxylation by hemoglobin and microsomal cytochrome P450 using isotopes, *Toxicology Letters* **70:** 147–153, 1994.

Byczkowski, J.Z. and A.P. Kulkarni. Oxidative stress and asbestos. In: *Environmental Oxidants,* Nriagu, J.O. (ed), John Wiley & Sons, New York, pp. 459–474, 1994.

Dodd, D.E., B.O. Stuart, S.J. Rothenberg, M. Kershaw, P.C. Mann, J.T. James, and C.W. Lam. Acute, 2-week, and 13-week inhalation toxicity studies on dimethylethoxysilane vapor in Fischer 344 rats, *Inhalation Toxicology* **6:** 151–166, 1994.

Joseph, P., S.N. Srinivasan, **J.Z. Byczkowski,** and A.P. Kulkarni. Bioactivation of benzo(a)pyrene-7,8-dihydrodiol catalyzed by lipoxygenase purified from human term placenta and conceptual tissues, *Reproductive Toxicology* **8:** 307–313, 1994.

Kulkarni, A.P. and **J.Z. Byczkowski.** Effects of transition metals on biological oxidations. In: *Environmental Oxidants,* Nriagu, J.O. (ed), John Wiley & Sons, New York, pp. 475–496, 1994.

Tyl, R.W., B. Ballantyne, L.C. Fisher, D.L. Fait, T.A. Savine, I.M. Pritts, and **D.E. Dodd.** Evaluation of exposure to water aerosol or air by nose-only or whole-body inhalation procedures for CD-1 mice in developmental toxicity studies, *Fundamental and Applied Toxicology* **23:** 251–260, 1994.

APPENDIX C

TOXIC HAZARDS RESEARCH UNIT GUEST SPEAKERS

DATE	TITLE	PRESENTER
10 December 1993	The Historical Practice of Health Risk Assessment in the United States: How That Experience Can Benefit Local Cleanup Initiatives	M. Gargas
14 February 1994	Electrical Analog Models of Plant-Water Relations	D. Alm
25 February 1994	Biomathematical Modeling in Life Sciences Research	R. Collins
4 May 1994	Quality Assurance for Life Science Research	P. O'Brien Pomerleau
14 July 1994	Analytical Chemistry for TCE and Metabolites	W. Brashear and M. Ketcha
16 August 1994	Cellular Electrophysiology in Neurotoxicology	J. Lin
1 September 1994	Biologically Based–Dose Response Modeling for TCE and its Metabolites	H. Clewell